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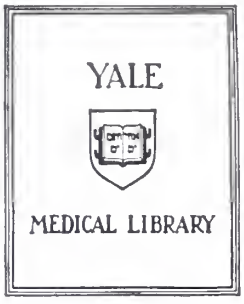
PROTEIN PHOSPHORYLATION AND DEPHOSPHORYLATION IN THE PARIETAL CELL:

The role of the regulatory subunit of Type II cAMP-dependent protein kinase in histamine-stimulated acid secretion

Valerie Anne Asher

Yale University

1990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PROTEIN PHOSPHORYLATION AND DEPHOSPHORYLATION IN THE PARIETAL CELL:

The role of the regulatory subunit of Type II cAMP-dependent protein kinase in histamine-stimulated acid secretion

A Thesis Submitted to the Yale University School of Medicine in Partial Fulfillment of the Requirements for the Degree of Doctor of Medicine

by

Valerie Anne Asher

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ABSTRACT

PROTEIN PHOSPHORYLATION AND DEPHOSPHORYLATION IN THE PARIETAL CELL: THE ROLE OF THE REGULATORY SUBUNIT OF TYPE II CYCLIC AMP-DEPENDENT PROTEIN KINASE IN HISTAMINE-STIMULATED ACID SECRETION

Valerie Anne Asher

1990

The role of cAMP in mediating histamine-stimulated parietal cell secretion is well established. Traditional views have suggested that the actions of cAMP are mediated through the activation of cAMP-dependent protein kinase (CAPK). The data presented here suggest a new model for cAMP-mediated acid secretion in which dephosphorylation of the regulatory subunit of Type II CAPK (RII) plays a critical role.

Isolated rabbit parietal cells (85 ± 5% pure) were incubated with either 100 μM cimetidine (unstimulated) or a combination of 100 μM histamine and 10 μM forskolin (maximally stimulated) for 45 min. The cells were then homogenized and subcellular fractions were prepared to localize phosphorylation changes to specific intracellular compartments. Differential centrifugation at 40xg, 4000xg, 14,500xg, and 48,500xg was used to prepare the subcellular fractions which were then characterized and assayed for endogenous kinase activity using [gamma-^{32}P]-ATP as a phosphate donor. Phosphorylated proteins were then resolved using SDS-PAGE and autoradiography.

A 52 kDa protein (pp52) was found to be phosphorylated in the stimulated apical membrane fraction and the unstimulated cytosolic fraction. This activity was

extremely rapid; pp52 was 100% phosphorylated within 45 sec. The kinase was dependent on Mg^{2+} and ATP ($K_{½}$ 3 μM) but could also use GTP as a phosphate donor ($K_{½}$ 10 μM). The addition of 10 μM cAMP to protein samples from stimulated apical membrane fractions abolished the phosphorylation of pp52. This inhibitory effect of cAMP was found to be due to the presence of a membrane-associated phosphatase activity that completely dephosphorylated pp52 within 120 sec. Although this phosphatase activity was not inhibited by Zn^{2+}, Mn^{2+}, pyrophosphate, or NaF, it was inhibited by the phosphatase inhibitor okadaic acid, at a concentration that suggested it was mediated by a Type 1 phosphatase. Isoelectric focusing, phosphoamino acid analysis, cAMP-binding studies, and immunoprecipitation identified the 52 kDa proteins in both membrane and cytosol as the regulatory subunit of Type II CAPK. Immunoprecipitation also suggested that the proteins found in membrane and cytosol were two different subtypes of RII.

Whole cell secretion studies also supported the hypothesis that a phosphatase plays a critical role in histamine-stimulated acid secretion. Histamine-stimulated [^{14}C]aminopyrine ([^{14}C]AP) uptake was measured in isolated rabbit parietal cells in the presence and absence of okadaic acid. Okadaic acid inhibited histamine-stimulated [^{14}C]AP uptake with an ID_{50} of approximately 10 μM. In contrast, H-8, an inhibitor of CAPK did not affect histamine-stimulated [^{14}C]AP uptake, suggesting the CAPK may not play a major role in histamine-stimulated acid secretion. These results suggest that the histamine-stimulated increase in cAMP may stimulate processes other than activation of cAMP-dependent protein kinase.

ACKNOWLEDGEMENTS

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I have drawn on the expertise of several people to produce this thesis in its final form. To Dr. David Berkowicz, the spirit medium of the MacIntosh, I owe my graphics and gratitude. Lillemor Wallmark printed all of the photographs. Anthony Giudice and Carol Russo provided administrative support.

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LIST OF ABBREVIATIONS

AP	aminopyrine
ADP	adenosine diphosphate
ATP	adenosine triphosphate
BSA	bovine serum albumin
C	catalytic subunit of cAMP-dependent protein kinase
cAMP	cyclic adenosine 3′:5′-monophosphate
CAPK	cAMP-dependent protein kinase
cGMP	cyclic guanosine 3′:5′-monophosphate
CK II	casein kinase II
DBcAMP	dibutyryl cAMP
DEAE	diethylaminoethyl
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylaminediaminetetraacetic acid
EGF	epidermal growth factor
GSK 3	glycogen synthase kinase 3
GTP	guanosine triphosphate
HEPES	N-2-hydroxyethylpiperazine-N'-ethanesulfonic acid
IBMX	isobutyl-1-methylxanthine
IEF	isoelectric focusing
IF	intrinsic factor
kDa	kilo Dalton

MAP-2	microtubule-associated protein 2
MES	2-[N-morpholino]ethanesulfonic acid
PGE_2, PGI_2	prostaglandin E_2, prostaglandin I_2
PKC	protein kinase C
R	regulatory subunit of CAPK
RI	regulatory subunit of Type I CAPK
RII	regulatory subunit of Type II CAPK
RII-B	RII, brain (neural) subtype
RII-H	RII, heart (non-neural) subtype
RIPA	radioimmune precipitant assay
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TPA	12-O-tetradecanoylphorbol-13-acetate

INTRODUCTION

While the formation of gastric and duodenal ulcers is probably determined by the balance between destructive and protective factors, it has often been observed that ulcers are seen only in the presence of acid. Thus the control of acid secretion is of considerable interest in the treatment of ulcer disease. However the mechanism of acid secretion is still poorly understood; thus drug therapies have been directed primarily at the first and last steps in acid secretion.

The first step in acid secretion involves hormone-receptor interactions at the cell surface. Known stimuli of acid secretion include histamine, acetylcholine, and gastrin. The Type II histamine (H_2) antagonists competitively bind to histamine receptors on the cell surface, thus preventing the endogenous secretagogue from initiating acid secretion. Because the various pathways that lead to acid secretion are interdependent, these drugs also decrease the amount of acid secreted in response to gastrin and cholinergic agents[1]. Anticholinergic drugs such as atropine work in a similar fashion to decrease gastric acid secretion. However, because of their serious side effects, more specific antimuscarinics such as the M1 antagonist pirenzapine are preferable[2], though less efficacious. The final step in acid secretion is the actual secretion of protons. Omeprazole, a substituted benzimidazole, specifically inhibits the H^+/K^+-ATPase that is ultimately responsible for pumping protons into the lumen of the stomach[2].

While the H_2 receptor blockers have made the treatment of peptic ulcer disease far more effective, some forms of gastric and duodenal ulceration, such as the stress ulcers sometimes seen in patients with shock, burns, sepsis, or

trauma, may not be amenable to conventional medical therapies[3]. This may reflect the fact that agents other than acid, such as pepsin, may be involved. However, a better understanding of the cellular physiology of acid secretion might enable us to treat such patients more effectively. In order to do this the molecular mechanism of acid secretion must be defined and the subcellular regulatory pathways delineated. This project represents an attempt to illuminate this area by examining the role of protein phosphorylation in the secretion of acid by the parietal cell.

The secretagogues that stimulate acid secretion also stimulate a number of so-called "second messengers," suggesting that several convergent pathways control acid secretion. These second messengers, such as cyclic adenosine 3′:5′-monophosphate (cAMP) and calcium, are thought to regulate intracellular processes by activating protein kinases and phosphatases. These enzymes alter the activities of essential protein substrates by adding or removing phosphate groups. It is therefore reasonable to postulate that the phosphorylation state of a key substrate may play a role in the control of acid secretion.

CHAPTER ONE

ACID SECRETION IN PERSPECTIVE

THE STOMACH

While the work presented here has focused on gastric acid secretion, the stomach serves many functions. Muscular walls allow it to expand and act as a reservoir for food, while peristaltic contractions serve to mix chyme with gastric secretions and to empty the gastric contents into the duodenum. The stomach is also an endocrine organ, for the antral mucosa contains G-cells that produce gastrins, a group of hormones that stimulates acid and pepsinogen secretion, gastric and intestinal motility, increased pressure in the lower esophageal sphincter, and bicarbonate secretion by the pancreas and liver. The exocrine secretions of the stomach include HCl, pepsinogen, intrinsic factor, and mucus. The secretion of gastric acid lowers the pH of the gastric contents. This acidification serves several purposes. Gastric HCl may sterilize the contents of the stomach. It also promotes the conversion of pepsinogen to pepsin and provides the low pH necessary for pepsin to catalyze the breakdown of proteins.

ACID SECRETION: HISTORY

Given the complexity of gastric function, it is not surprising that the study of gastric acid secretion has been enmeshed in controversy. In the past, scientists disagreed about whether the stomach secreted acid and if so, which acid it produced. Only recently has the parietal cell been identified as the site of acid production. Thus it now remains to identify the intracellular processes that mediate

gastric HCl secretion.

The history of the study of acid secretion has been extensively reviewed in an excellent review article by Modlin and is briefly summarized here[4]. Early Greek theories of digestion recognized neither the role of the stomach nor the function of acid in digestion. Not until the sixteenth century did Paracelsus assert that the stomach contains acid and that it is necessary for digestion. He believed that by drinking acidic spa waters one introduced acid into the stomach. Jean Baptiste von Helmont and Sylvius also held to this chemical theory of digestion but did little to further understanding of the nature or source of gastric acid. Indeed von Helmont believed the spleen to be the source, while Sylvius maintained that the digestive processes of the stomach were mediated by saliva. These contradictory chemical theories were collectively challenged by a second school of thought that maintained that the stomach's main function was mechanical.

In the eighteenth century, experimental science blossomed, and investigators such as Reaumur, Reuss, Gosse, Stevens, Spallanzani, Scopoli and Carminati studied digestion and the contents of the stomach in both humans and animals. Scopoli and Spallanzani produced evidence to suggest that the stomach contained hydrochloric acid. This was confirmed definitively by William Prout in 1823, when he presented his paper "On the Nature of Acid and Saline Matters Usually Existing in the Stomach of Animals" to the Royal Society of London. In this work he showed evidence that hydrochloric acid is found in the gastric juice of many different species. He was also able to quantify his data, showing the total amount of hydrochloric acid present and relating it to dyspeptic symptoms. This

work predated that of William Beaumont, who published his first experiments in 1826. Nonetheless, complete acceptance of Prout's theory did not come until this century. Various investigators attempted to show that acetic, butyric, lactic, and phosphoric acids were secreted by the stomach. Not until 1930 did Dodds and Robertson confirm that hydrochloric acid was the sole acid produced by the stomach.

Beginning with Pavlov's studies on vagal control of gastric secretion, scientists began to investigate neural control of acid secretion. At the same time other researchers began to elucidate the humoral regulation of the gastrointestinal tract, identifying both histamine and gastrin as important regulators of gastric acid production. However the exact source of gastric acid remained unknown.

At the beginning of the nineteenth century Matthias Schleiden and Thomas Schwann opened the world of cell biology with the theory that all life is composed of cells. Over the next century, the different gastric cell types, including the parietal cell, were identified. The parietal cell took its name from the Latin word for wall, based on its tendency to bulge outward from the wall of the gastric gland[5]. It is now also known as the oxyntic cell, a name given because of its ability to produce acid[5]. The morphology of the parietal cell was described, with its numerous, large mitochondria, cytoplasmic tubulovesicles, and extensive invaginations of the apical membrane known as "intracellular" canaliculi despite their apparent continuity with the gland lumen[6].

In 1858, Claude Bernard used chemical and microscopic methods in an attempt to determine the site of acid formation. Using a precursor of Prussian

blue, a colored pH indicator, he injected rabbits, then removed their stomachs for microscopic examination. He found the blue color indicative of low pH only in the superficial layer of the mucosa; it stained neither the gastric glands nor the canaliculi of the parietal cell. Mistakenly, he took this as evidence that the stomach secreted a neutral substance that underwent further reaction to become acidic within the lumen of the stomach[7].

Later investigators found circumstantial evidence to suggest that the parietal cell was indeed the source of gastric HCl. Acid is found only in stomachs that contain parietal cells, and the amount produced is proportional to the number of parietal cells present[5,8]. The presence of acid in the fetal stomach was also found to be temporally related to parietal cell differentiation[9]. The unique morphology of the parietal cell seemed well suited for this purpose, with its abundant, large mitochondria to provide energy and its extensive, dynamic system of canaliculi. These were believed to be a secretory apparatus that cycled between the resting and the stimulated states. A number of investigators looked for further evidence that the parietal cell was the source of gastric acid. Bradford and Davies used 26 pH indicator dyes to study isolated amphibian and mammalian gastric mucosa. They found that in histamine-stimulated tissue, the contents of the intracellular canaliculi had a pH of less than 4.8[5,10]. Kominick used silver to precipitate chloride and found precipitates in the glandular lumen and vesicles of parietal cells. This physical proximity was taken as further evidence that parietal cells are the source of gastric HCl[5]. Later studies looked at isolated rabbit gastric glands with interference, electron, and fluorescence microscopy. Parietal cells in histamine-

stimulated gastric glands formed intracellular spaces consistent with the expanded canalicular network previously described in stimulated cells. The use of the pH-dependent fluorescent indicator acridine orange confirmed that the spaces were indeed acidic[11,12]. Another line of investigation developed when HCl secretion was found to be dependent on a mechanism requiring ATP and K^+: the H^+/K^+-ATPase. A monoclonal antibody developed against the catalytic subunit of this enzyme recognized only one kind of cell in the gastric mucosa, the parietal cell[13]. This provided substantial support for the theory that the parietal cell is the source of gastric acid.

PARIETAL CELL MORPHOLOGY

The simple columnar epithelium that lines the mammalian stomach is interrupted by gastric pits, invaginations of the mucosa that lead to one or more tubular gastric glands. The gastric glands of the fundus and corpus, known as oxyntic glands, contain several types of cells: the oxyntic or parietal cell, the chief cell, the mucus neck cell, as well as endocrine cells (Figure 1). The parietal cell is the most numerous cell of the corpus, making up about 32% of the corpus mucosa[5]. The normal human stomach contains approximately one billion parietal cells[9]. They are usually found in the neck and isthmus regions of the oxyntic gland but can also be found along the base.

The parietal cell is an oval to pyramidal cell that may be as large as 25 μM. Several features make it morphologically distinctive[5]. First, it contains abundant, large mitochondria to provide the energy necessary for the proton pump. Second,

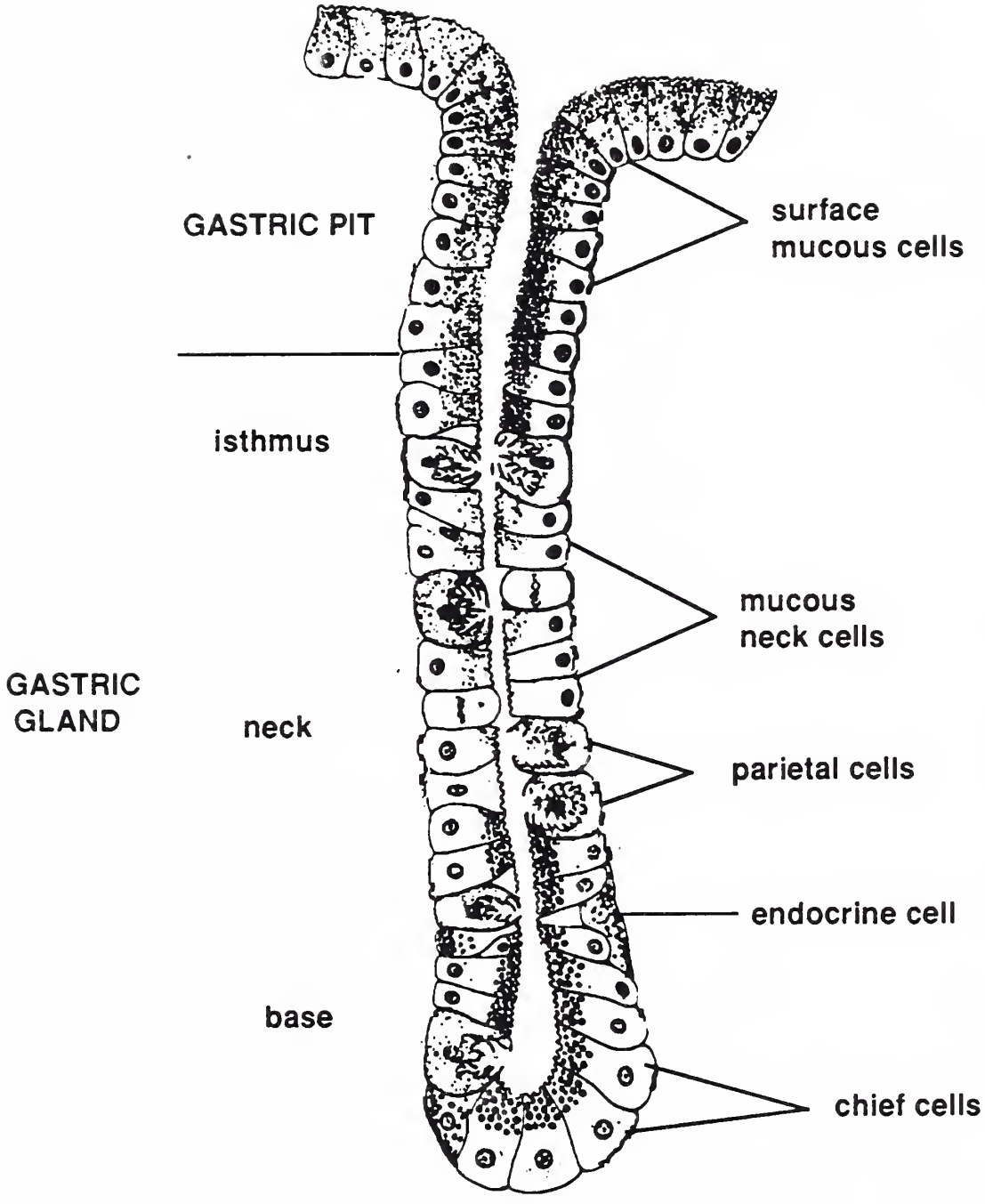

FIGURE 1: The gastric gland.
Simplified drawing of a tubular gastric gland from the corpus of a mammalian stomach showing the basic cell types: mucous neck cells, parietal cells, chief cells, and parietal cells. Parietal cells are concentrated in the neck and isthmus of the gland, although they are also found along the base. (Adapted from ref. 5).

in the resting state, the cytoplasm is filled with smooth, membranous tubulovesicular structures (Figure 2). Lastly, the cell is penetrated by canaliculi that are continuous with the apical membrane of the cell and communicate with the oxyntic gland lumen. In the unstimulated cell, the canaliculi are quite small and possess short, stubby microvilli as does the apical membrane of the cell. When the parietal cell is stimulated with secretagogues that promote acid secretion, the canalicular system expands and the microvilli grow longer and more numerous, increasing the apical surface area by a factor of four or more. At the same time the tubulovesicular membranes disappear from the cytoplasm (Figure 2). When secretion stops, the microvilli of the canalicular system return to their original state, while the tubulovesicles reappear in the cytoplasm[14,5]. Morphometric studies have shown that the increase in the apical surface area corresponds to a decrease in that of the tubulovesicles[15].

Two hypotheses have attempted to explain these morphological changes[16,17,18]. The osmotic flow or expansion hypothesis contends that the tubulovesicular membranes are continuous with the apical membrane but that in the resting cell the tubulovesicles are supercollapsed. They expand in response to net ionic and volume flow, thus creating the apparent structural changes in the cell. However experiments that induced the accumulation of water and salt within tubulovesicles did not lead to enlargement of the apical membrane[17]. It therefore seemed that osmotic change alone was not sufficient to bring about the morphological changes associated with secretion. No further data has been produced in support of the osmotic flow theory.

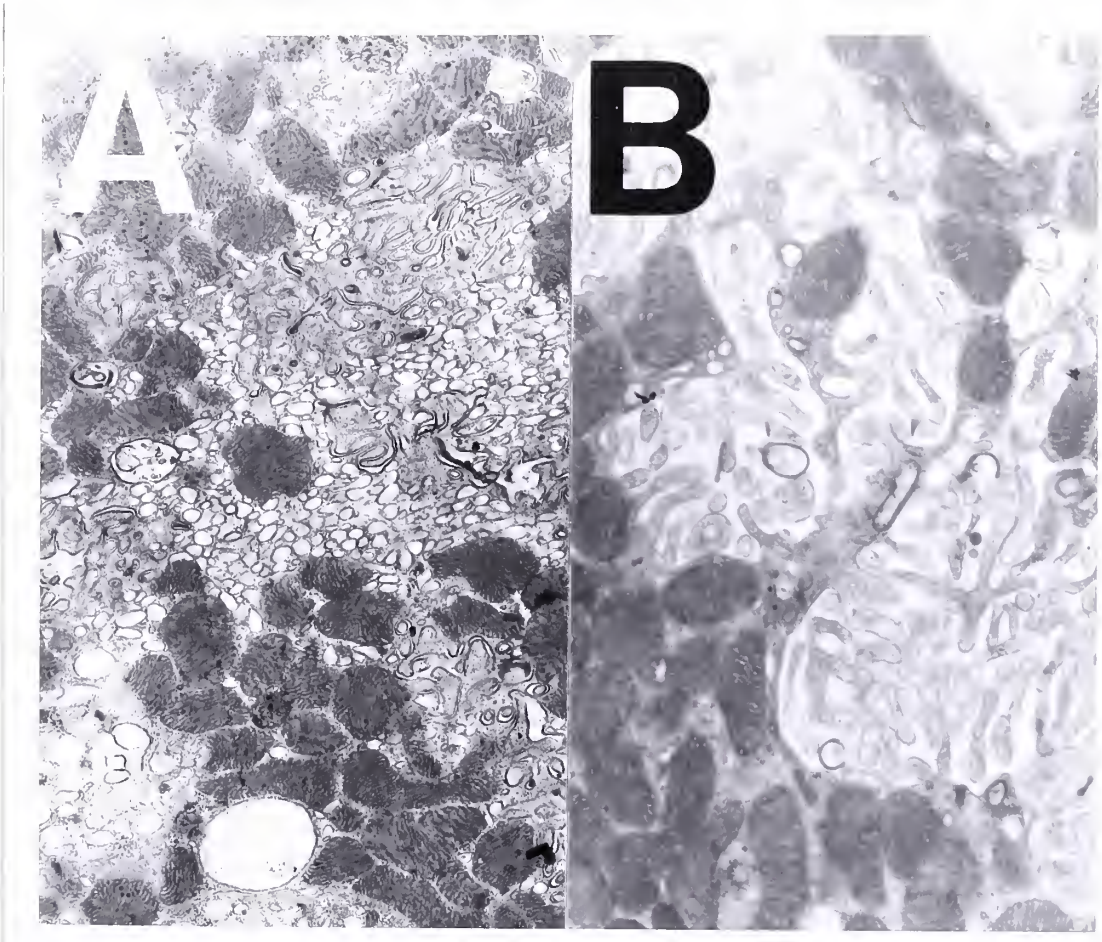

FIGURE 2: Electron micrographs of resting and histamine-stimulated parietal cells.

Electron micrographs (10,000 x magnification) were prepared from isolated rabbit parietal cells prepared as described in Chapter 3. Histamine-stimulated cells were incubated with 10 μM histamine for 45 min. In the resting parietal cell (A), numerous tubulovesicles fill the cytoplasm. Large numbers of mitochondria are also present. In the stimulated parietal cell (B), the tubulovesicles have disappeared from the cytoplasm and the canalicular network has become more elaborate. (Electron micrographs courtesy of Dr. James Goldenring, Yale University, New Haven, CT).

Far more credible is the membrane recycling or fusion hypothesis, which proposes that the tubulovesicles fuse with the apical membrane upon stimulation of the cell. When the cell returns to the resting state these membranes are somehow retrieved to form tubulovesicles[19]. Several pieces of evidence support this theory. Antibodies directed against the H^+/K^+-ATPase localized the enzyme to the tubulovesicles of the resting cell, with less of the enzyme being found in the apical membrane. In contrast, in the stimulated cell, the H^+/K^+-ATPase was predominately found in the apical membrane[13], suggesting that the movement of the H^+/K^+-ATPase depends upon the fusion of tubulovesicles with the apical membrane. Freeze-fracture electron microscopy has also shown that upon histamine stimulation, the particle distribution of the apical membrane changes, becoming more like that of the tubulovesicles[19]. This suggests that the tubulovesicular membrane has become part of the apical surface. Other studies of fixed cells with macromolecular tracers or extracellular surface stains have failed to show any direct communication between the cell surface and the tubulovesicles. However when macromolecular tracers were incubated with living cells, prior to fixation, they showed up within the tubulovesicles[16]. This is consistent with the fusion hypothesis in that there is no direct communication between the cell surface and tubulovesicles, but that a pathway does exist for the movement of materials between the apical membrane and the tubulovesicles.

Changes in the cytoskeletal architecture of the parietal cell parallel the changes in membrane structure. Actin-like filaments are found in the microvilli, while other studies have shown microfilaments and microtubules beneath the

microvilli[20,14]. Double labelling of cells with antibodies against actin and spectrin showed striking evidence of carbachol-stimulated cytoskeletal reorganization corresponding to changes in the membrane structure of stimulated cells[21]. Disruption of actin filaments and microtubules also disrupts parietal cell function. When cytochalasin, a drug which prevents the elongation of actin filaments, is added to piglet mucosa, acid secretion is inhibited[22]. Similarly, when guinea pig parietal cells were treated with colchicine and vinblastine, histamine-stimulated acid production was impaired[23]. These data suggest that cytoskeletal elements may mediate both the fusion of tubulovesicles with the apical membrane in the stimulated cell and their retrieval when the cell returns to the resting state.

PARIETAL CELL PHYSIOLOGY

Methods of study

In vivo a number of stimuli cause gastric acid secretion: the sight, taste, smell and thought of food, gastric distention, proteins and amino acids in the stomach, chyme in the duodenum, vagal stimulation, acetylcholine, histamine, and gastrin[24]. Because of the complexity of this system, neither in vivo studies nor those using intact gastric mucosa shed much light on the direct effectors of parietal cell acid secretion. For this purpose, cell separation techniques, using enzymatic digestion and centrifugation, have been developed to facilitate the study of viable gastric glands[25] and isolated gastric cells. Because of the large size of the parietal cell, techniques such as counterflow elutriation and centrifugation on density gradients can be used to prepare cells that are up to 90% pure[26]. More

recently a procedure has been developed that allows the culture of highly enriched parietal cells, making long-term studies feasible for the first time[27].

Studies of dispersed gastric glands and cells have one important disadvantage. With the loss of mucosal polarity, it is no longer possible to measure proton secretion directly, since the basolateral membrane secretes a neutralizing bicarbonate ion[26]. Therefore indirect methods have been developed to permit the study of parietal cell function in vitro[11,26,28].

The energy requirements of the parietal cell provide one way to assess secretory activity. The H^+/K^+-ATPase, the proton pump, requires ATP. This ATP is provided by the abundant mitochondria that occupy 30 to 40% of the parietal cell cytoplasm. In order to produce ATP, the mitochondria consume oxygen. Thus oxygen consumption provides an indirect measure of secretory activity. A similar protocol measures the formation of $^{14}CO_2$ from [^{14}C] glucose to determine metabolic activity[26].

Another measure of secretory activity makes use of the profound morphologic transformation of the stimulated parietal cell. This change can be observed in living cells using either differential interference-contrast microscopy (Nomarski optics) or fluorescent microscopy. The latter can be used with acridine orange, a weak base that accumulates in acidic spaces and emits a red fluorescence at low pH[11,26].

The tendency of weak bases to accumulate in membrane-bound acidic spaces is also used in another method. A radioactive weak base such as [^{14}C]aminopyrine ([^{14}C]AP), which has a pKa of 6, will accumulate in the acidic

spaces of actively secreting parietal cells. Acid sequestration can be quantified by simply measuring the radioactivity that remains associated with washed parietal cells after incubation with [^{14}C]AP[26,28]. This method is probably the most widely used in the study of parietal cell secretory activity.

While these methods have helped to overcome one problem encountered in the study of acid secretion, others exist that are not so easily solved. Particularly troubling are the variations in parietal cell function found among different species. For example, in some mammalian species, including rabbit and man, the parietal cell secretes intrinsic factor (IF) as well as HCl[29], whereas in other species the chief cell is responsible for secreting IF. In nonmammalian vertebrates, a single cell type, the oxyntopeptic cell secretes both pepsinogen and acid[5]. It has therefore been difficult to establish whether there exists a generalized mechanism of acid secretion with some minor variations between species. However most reports support a common mechanism of receptor-mediated proton secretion effected by the H^+/K^+-ATPase proton pump.

The regulation of acid secretion in vivo

In vivo studies show that paracrine, neurocrine, and endocrine pathways influence gastric acid secretion. When the isolated gastric gland or parietal cell is studied *in vitro*, these pathways appear to correspond to three recognized secretagogues: histamine, acetylcholine, and gastrin (Figure 3). Histamine is found in the mast cells of the gastric mucosa, although it is possible that a second cell type in the gastric mucosa also produces histamine. The parasympathetic nerve

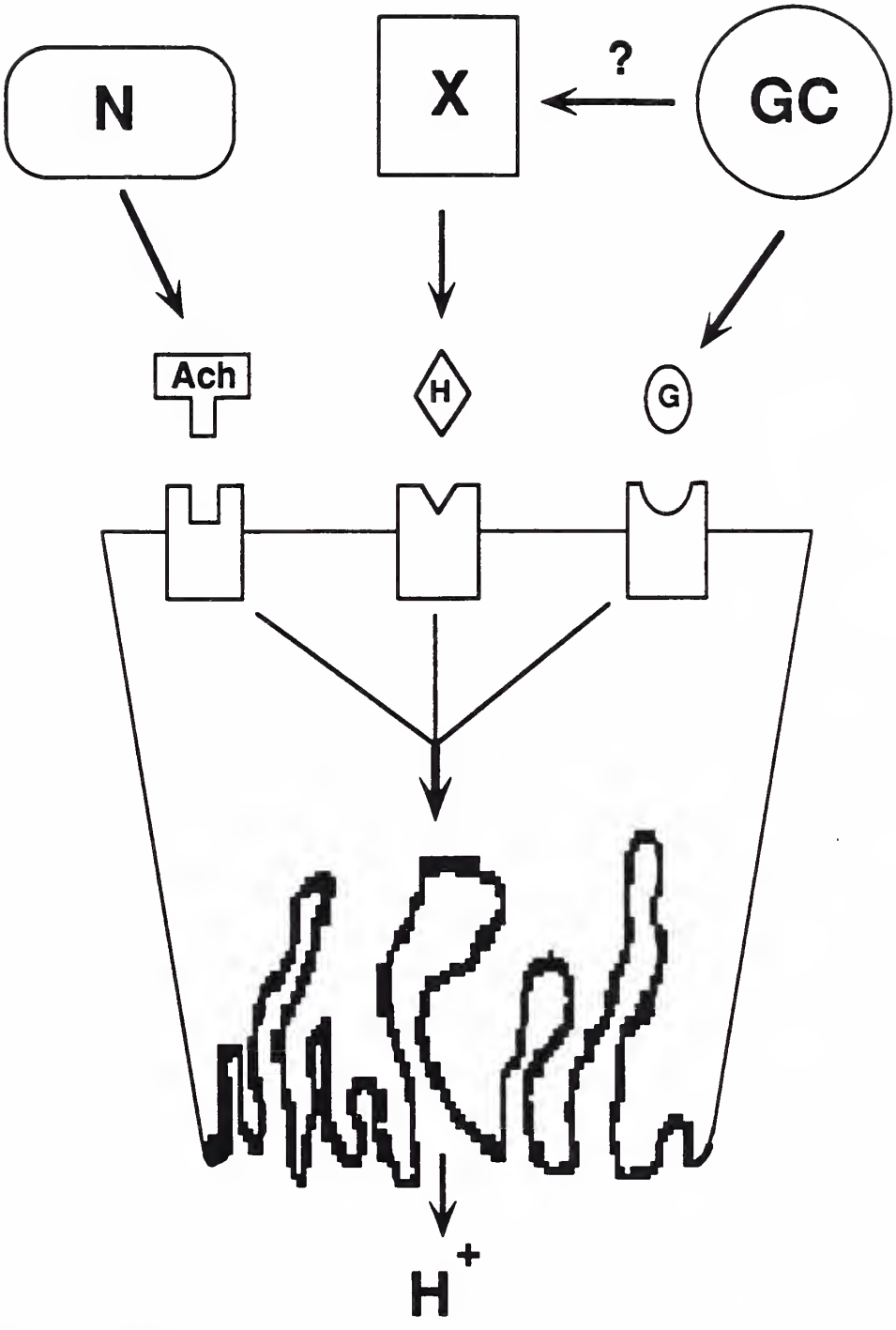

FIGURE 3: Regulatory pathways of the parietal cell.
Histamine, acetylcholine, and gastrin appear to stimulate the parietal cell; however gastrin receptors have been demonstrated only on canine parietal cells. In other species gastrin may act indirectly by stimulating the release of histamine. It is not yet clear which cell type in the gastric mucosa is the source of histamine. N - neuron; X - histamine-producing cell; GC - G cell; Ach - acetylcholine; H - histamine; G - gastrin.

terminals release acetylcholine. Gastrin, a peptide hormone, is released by the gastric endocrine G cells.

Histamine stimulation of acid secretion

There is a large body of evidence to support the assertion that histamine stimulates gastric acid secretion. Berglindh et al.[25,28] first showed that histamine stimulated oxygen consumption and [^{14}C]aminopyrine uptake in a dose-dependent fashion in isolated rabbit glands with an ED_{50} of 3 x 10^{-6}M, reaching a maximum after about 30 minutes. Histamine stimulation of parietal cells has also been demonstrated in cells from many other species, including pig[30], dog[31], rat[32], guinea pig[33], frog[26], and human[34]. However there are differences between species in the magnitude of the response to secretagogue[35]. For example histamine-stimulated [^{14}C]aminopyrine uptake is greater in rabbit parietal cells than in those of the dog[26]. The response to histamine appears to be mediated through a typical H_2 receptor. The H_2 receptor antagonist cimetidine was found to be a competitive inhibitor of histamine-stimulated rabbit gastric gland secretion[36]. While the H_1 receptor antagonist mepyramine successfully inhibited histamine-stimulated [^{14}C]aminopyrine accumulation in canine cells, it did so at a concentration 10^5 times higher than that required for H_1 receptor blockade[31]. Thus histamine and its antagonists appear to act through an H_2 receptor.

Histamine stimulation of parietal cells also increases the concentration of cyclic adenosine monophosphate (cAMP) in many species, including rabbit[36] and dog[37]. This was enhanced in the presence of the phosphodiesterase inhibitor 3-

isobutyl-1-methyl xanthine (IBMX)[38]. Several facts indicate the involvement of cAMP in parietal cell acid secretion. First, the increase in cAMP correlated with histamine stimulation of parietal cell function as measured by oxygen consumption and [^{14}C]aminopyrine accumulation[38]. Second, the action of histamine can be enhanced by the use of IBMX, which prevents the degradation of cAMP by cAMP phosphodiesterase[26]. Third, the use of forskolin, a specific activator of adenylate cyclase, the enzyme that converts ATP to cAMP, increases both parietal cell activity and intracellular cAMP[39]. Lastly, the use of the cell-permeant cAMP analogues dibutyryl cAMP (DBcAMP) and 8-bromo-cAMP directly stimulate [^{14}C]aminopyrine accumulation in isolated canine parietal cells[31].

While the study of histamine-stimulated parietal cell activity has focused on the role of cAMP, a second system may also play a part. Studies using the calcium-sensitive dyes quin2 and fura-2 have shown that histamine causes an increase in the intracellular calcium ($[Ca]_i$)[40,41]. Histamine stimulation led to a calcium spike within 5 seconds followed by a plateau level that persisted until the removal of agonist or the addition of antagonist. The H_2 receptor antagonist cimetidine specifically blocked this reaction. This increase does not appear to be mediated by cAMP, since addition of DBcAMP and IBMX did not affect the intracellular calcium concentration[42,43]. However forskolin was found to increase cytosolic free Ca^{2+} in rabbit gastric glands[40] and pig parietal cells[30]. The calcium spike occurred in the presence of calcium channel blockers. Thus it seems to represent the release of calcium from an intracellular store. In contrast, the plateau that appears after the spike appears to depend on the influx of extracellular calcium

across the plasma membrane[43]. The role of the Ca^{2+} signal in histamine-stimulated acid secretion is unclear since chelation of neither extracellular [43,44] nor intracellular[44] Ca^{2+} affected histamine-stimulated uptake of [^{14}C]aminopyrine in rabbit parietal cells.

Cholinergic stimulation of acid secretion

Cholinergic agonists such as carbachol have also been shown to affect parietal cell secretory activity[45,28]. When carbachol was added to isolated rabbit glands, oxygen consumption, a marker for acid secretion, increased, reaching a maximum at 15 to 20 minutes and slowly declining from that point[28]. This is in marked contrast to the pattern seen with histamine stimulation, where peak parietal cell activity is sustained at a maximal plateau. The secretory response to carbachol in rabbit glands is also weaker than that seen with histamine[28]. Some differences exist between species. In canine parietal cells, carbachol stimulates a sustained response that is greater than that of either histamine or gastrin[31]. The specificity of the response to cholinergic agonists has been shown by the fact that it is competitively inhibited by atropine but unaffected by the H_2 receptor blocker cimetidine[31].

Carbachol appears to act independently of cAMP. The stimulatory effect is seen without a corresponding increase in intracellular cAMP[38]. However, like histamine, carbachol stimulates an increase in the intracellular calcium concentration of the parietal cell. This increase can be blocked by the muscarinic antagonist atropine[43]. The addition of carbachol leads to the efflux of calcium from

intracellular stores, causing a calcium spike followed by a plateau phase that seems to depend on the influx of extracellular calcium[43]. Because of calcium's known role as a second messenger[46], it is tempting to speculate that it plays a role in acid secretion. However it has been difficult to prove this connection. It has not been possible to use Ca^{2+} ionophores to study parietal cell proton secretion, because they also interfere with H^+ concentrations and thereby impair function of the proton pump[47]. However there is some data to suggest that calcium does play a part in acid secretion. In medium with a low concentration of Ca^{2+} (10^{-5}M) aminopyrine accumulation by acetylcholine-stimulated rabbit gastric glands is decreased by 70%[42]. Even more convincing is the fact that use of an intracellular calcium chelator (1,2-Bis(2aminophenoxy)ethane-N,N,N',N'-tetraacetic acid acetoxy methyl ester) eliminated both the carbachol-stimulated calcium signal and carbachol-stimulated [^{14}C]aminopyrine accumulation in rabbit parietal cells[44].

Gastrin stimulation of acid secretion

The role of gastrin in stimulating parietal cell acid secretion is somewhat less clear than that of histamine or cholinergic agonists. Gastrin receptors have been demonstrated only on canine parietal cells using ^{125}I-gastrin as a probe[48]. In addition, parietal cells from different species respond very differently to gastrin. When first studied in isolated rabbit gastric glands, gastrin failed to induce increased respiratory activity, increased [^{14}C]aminopyrine accumulation, or quantifiable changes in morphology[28]. Subsequent studies of isolated canine parietal cells showed a small increase in parietal cell activity with gastrin stimula-

tion. Neither atropine nor cimetidine blocked this response, suggesting that it was mediated through a third receptor that was neither cholinergic nor histaminergic[31]. Proglumide, an inhibitor of cholecystokinin, inhibits the effect of gastrin on parietal cells but does not appear to affect responses mediated by other receptor types[48]. This probably reflects the fact that cholecystokinin, a peptide hormone, shares a common tetrapeptide sequence with gastrin at the carboxyl terminal. Gastrin-stimulation of [^{14}C]aminopyrine accumulation was seen when rabbit gastric glands were treated with the phosphodiesterase inhibitor IBMX or the sulfhydryl reducing agent dithiothreitol (DTT). This response was inhibited by cimetidine[49]. β-Mercaptoethanol, which enhances the histamine sensitivity of the rabbit parietal cell, also enhanced the gastrin response[26]. Since gastrin is known to release histamine from gastric glands, it is possible that its effects on the rabbit parietal cell are mediated by histamine and not by the direct effect on the parietal cell that has been noted in the dog[26].

While there are several different regulatory pathways modulating parietal cell stimulatory activity, they are clearly interdependent. Histamine, carbachol, and gastrin potentiate one another's actions, producing effects that are greater than the sum of their effects alone[50,51]. Forskolin, which increases the concentration of cAMP by activating adenylate cyclase, also potentiates the effect of carbachol[40], as does the cell-permeant cAMP analogue DBcAMP[52]. This suggests that the potentiating effects of secretagogues are mediated within the cell and not at the level of the receptor.

Inhibitors of acid secretion

In considering parietal cell physiology, it is also important to note inhibitors of parietal cell function. The competitive muscarinic and H_2 antagonists have already been mentioned, as has omeprazole, the H^+/K^+-ATPase inhibitor, which is a noncompetitive inhibitor of all parietal cell secretagogues. The prostaglandins PGE_2 and PGI_2 inhibit histamine-stimulated parietal cell function[53] as well as the histamine-stimulated increase in cAMP[37,54,55,56]. Somatostatin also acts noncompetitively to inhibit histamine- and gastrin-stimulated acid secretion in rabbit, rat, and guinea pig parietal cells[56,57,58]. Specific somatostatin receptors have also been identified on canine parietal cells[59]. Somatostatin also inhibits the histamine-stimulated increase in cAMP. It is possible that somatostatin acts by inhibiting adenylate cyclase[60]. Pancreastatin, a peptide hormone that shares some sequence homology with gastrin, inhibits both histamine- and carbachol-stimulated aminopyrine uptake in rabbit parietal cells[61]. While pancreastatin inhibited the histamine-stimulated increase in cAMP, it had no effect on the carbachol induced calcium spike[61]. The available data suggest that pancreastatin acts to inhibit parietal cell signal transduction at several different points[61]. The inhibitory effects of epidermal growth factor (EGF) have also been described and are equally complex. EGF inhibits both histamine- and carbachol-stimulated aminopyrine uptake in isolated rabbit parietal cells[62,63,64]. Like pancreastatin, it seems to exert its effect at multiple points in the signal transduction pathway[63].

PROTEIN KINASES AND PROTEIN PHOSPHORYLATION IN THE PARIETAL CELL

Stimuli of gastric parietal cell HCl secretion are also known to increase intracellular concentrations of cAMP and Ca^{2+}. In many systems these "second messengers" activate cellular enzymes known as protein kinases. Protein kinases are able to phosphorylate other proteins. The addition of a phosphate group to many proteins alters their biological activities. This mechanism is thought to play a role in regulating a wide variety of cellular processes[65]. Many investigators have therefore tried to delineate the role of protein kinases and protein phosphorylation in parietal cell acid secretion (Tables I and II.)

Protein kinases

Studies focusing on the role of cAMP in the parietal cell have concentrated on the cAMP-dependent protein kinase (CAPK). This kinase is a tetramer made of two identical regulatory subunits (R) and two identical catalytic subunits (C) (Figure 4). In the inactive form, the regulatory subunit is bound to the catalytic subunit. When cAMP is bound to the regulatory subunit, the tetramer disassociates and the two catalytic subunits are released as the active enzyme. There are two types of CAPK, known as Type I and Type II. These differ only in the regulatory subunit; the catalytic subunit is the same for both Type I and Type II CAPK.

Using isolated rabbit gastric glands Jackson et al.[66] identified two cytosolic cAMP binding proteins with molecular weights of 48 kDa and 58 kDa. These were

SOURCE	STIMULUS	INTRACELLULAR LOCATION	KINASE
Rabbit FGM	-	cytosol	cAMP dependent (unaffected by K^{+})
		membrane	cAMP dependent (decreased by K^{+})
Rabbit GG	-	cytosol	Zn^{2+}/Mn^{2+} dependent
Rabbit PC	-	cytosol, membrane	cAMP dependent
Rabbit PC	-	cytosol	Calmodulin dependent
Rabbit PC	-	?	Ca^{2+}/phospholipid dependent (PKC)
Rabbit PC	-	?	2 Casein Kinases
Rabbit PC	histamine	cytosol	cAMP dependent (type I)
Guinea Pig FGM	histamine	cytosol	cAMP dependent
Rabbit PC	forskolin	cytosol, membranes	cAMP dependent (type II)
Rabbit PC	carbachol	?	Ca^{2+} dependent PKC
Rat FGM	-	membrane	Ca^{2+}/Calmodulin dependent

TABLE I: Protein kinases found in gastric tissue and parietal cells.
For references, see text. Abbreviations: FGM - fundic gastric mucosa; GG - gastric glands; PC - parietal cells; PKC - protein kinase C.

SOURCE	SECRETAGOGUE	KINASE DEPENDENCE	INTRACELLULAR LOCATION	SUBSTRATE PROTEINS
Rabbit PC	histamine		cytosol	148, 130, 47, 43 kDa
			membrane	130, 51, 47 kDa
	none	cAMP	cytosol	many, esp.131, 97,84, 47, 42, 33 kDa
			membrane	many, esp 130, 42, 20, 22 kDa
Rabbit PC	histamine	cAMP	cytosol	30 kDa
Rabbit PC	histamine	cAMP	membrane	27, 40 kDa
Rabbit GG	histamine + forskolin		membrane	80, 120 kDa
Rabbit PC	carbachol	Ca^{2+}/PKC	membrane, cytosol	28, 36 kDa
		PKC	?	2x 66 kDa
Rabbit PC	none	calmodulin	cytosol	100 kDa
Rabbit GG	none	Zn^{2+}/Mn^{2+}	cytosol	33 kDa

TABLE II: Protein kinase substrates found in gastric tissue and parietal cells. For references, see text. Abbreviations: FGM - fundic gastric mucosa; GG - gastric glands; PC - parietal cells; PKC - protein kinase C.

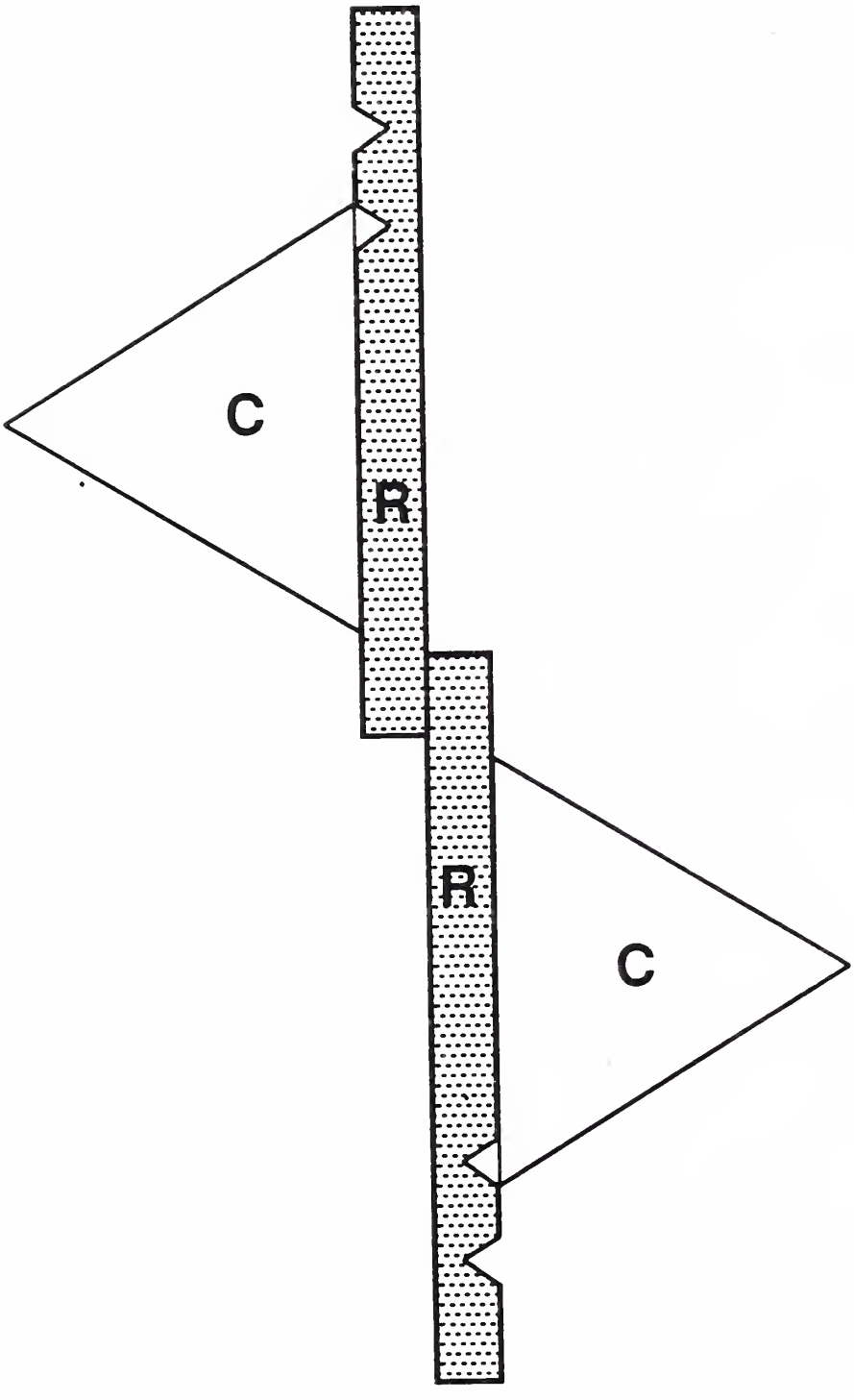

FIGURE 4: Structure of the cAMP-dependent protein kinase.
The kinase is a tetramer (R_2C_2) composed of two identical regulatory subunits (R) and two identical catalytic subunits (C). Each regulatory subunit has two cAMP binding sites.

found to correspond to two cytosolic cAMP dependent protein kinases thought to be Type I and Type II CAPK. When the glands were stimulated with histamine, binding of the azido-cAMP ligand decreased 32 to 44%. This is consistent with the hypothesis that when histamine increases endogenous cAMP, it binds to the regulatory subunit of CAPK, leaving fewer binding sites available for the exogenous ligand. Theoretically this should lead to increased CAPK activity in stimulated cells, and in fact several investigators have demonstrated this[67,68,69,70,71]. Histamine stimulation increased the activity of CAPK in both rabbit and guinea pig fundic mucosa[67,68,69]. The activation of CAPK was also found to reflect the tissue concentration of cAMP[70]. A more detailed study of CAPK activity in isolated parietal cells reported that the Type I isozyme was found predominately in the cytosolic fraction while the Type II isozyme was found in both particulate and cytosolic fractions[71]. Histamine stimulation of these cells was shown to activate only the cytosolic Type I kinase[71]. In contrast, forskolin was found to stimulate both forms of CAPK[71]. These data suggest that Type I and Type II CAPK are compartmentalized within the parietal cell and that histamine stimulation increases local concentrations of cAMP, thus selectively activating the Type I isozyme.

Other studies have attempted to identify putative substrates of CAPK in the parietal cell[72,73,74,75]. After preincubation with ^{32}P-labeled orthophosphate, secretagogue is added to the tissue preparation. Phosphorylation changes are evaluated by homogenizing the cells, using differential centrifugation to separate soluble and particulate material, and using one- and two-dimensional SDS-PAGE followed by autoradiography. A number of phosphoproteins have been identified in this

way, but it remains to see which of these actually play a role in acid secretion.

Using this methodology, Chew and Brown[72] have shown that stimulation with histamine increases the phosphorylation of 27 and 40 kDa proteins in low speed particulate fractions of parietal cells. Forskolin and cAMP analogues also increase the phosphorylation of these proteins, suggesting that a cAMP-dependent mechanism mediates the phosphorylation increase[72]. Oddsdottir et al.[73] have reported that histamine, forskolin, and 8-bromo-cAMP stimulated the phosphorylation of a 30 kDa cytosolic protein in rabbit parietal cells. Protein phosphorylation began to increase within one minute of the addition of secretagogue and reached a maximum at 15 minutes[73]. Malinowska et al. have found histamine-dependent increases in protein phosphorylation in both soluble and particulate fractions[74]. Phosphoproteins of 148, 130, 47, and 43 kDa were detected in the cytosolic fraction, while phosphoproteins of 130, 51, and 47 kDa appeared in the particulate fraction.

Urushidani et al. have done extensive work characterizing a membrane-bound phosphoprotein in gastric glands. They examined subcellular fractions of histamine-stimulated rabbit gastric glands and found 80 and 120 kDa proteins in the heavy particulate fraction[75]. This same fraction is also greatly enriched in H^+/K^+-ATPase activity, as measured by K^+-p-nitrophenyl phosphatase activity, in histamine-stimulated gastric glands[76]. Since H^+/K^+-ATPase activity can be used as a marker for the apical membrane in stimulated cells, this has been referred to as the apical-membrane rich fraction. The 80 kDa protein was also phosphorylated when glands were treated with DBcAMP, whereas neither carbachol-

stimulation nor chelation of intracellular calcium affected its phosphorylation state[77]. Like the H^+/K^+-ATPase, the 80 kDa protein is tightly membrane-associated[77]. The 80 kDa protein could also be phosphorylated in vitro by the addition of ^{32}P-ATP to the apical-membrane rich fraction. The addition of cAMP-dependent protein kinase inhibitor blocked this reaction, suggesting that CAPK is at least partly responsible for phosphorylating the 80 kDa protein[77]. Monoclonal antibodies against this 80 kDa protein have been used to show that within the gastric mucosa, it is found only in parietal cells[78]. Immunocytochemistry further revealed that the 80 kDa protein is localized to a fine, filamentous network within the parietal cell. This staining pattern is similar to that of F-actin. When the cells are stimulated with histamine, the distribution of the 80 kDa protein changes to a coarser, rugose pattern identical to that seen for immunolocalization of the H^+/K^+-ATPase[78]. The change in the staining pattern of the 80 kDa protein probably reflects the elaboration of the apical membrane that is seen in the stimulated cell.

Far fewer substrates for protein phosphorylation have been identified in carbachol-stimulated parietal cells. Carbachol-stimulation increased phosphorylation of two 66 kDa proteins with different isoelectric points and a 36 kDa protein[44]. Carbachol also induced a transient increase in the phosphorylation of a 28 kDa protein[44]. The 36 and 28 kDa proteins are found in the 100,000 x g supernatant and pellet, rather than the apical membrane fractions[44]. Several pieces of evidence support the hypothesis that the carbachol-stimulated calcium signal mediates the phosphorylation of these proteins. The calcium ionophore, ionomycin, was able to stimulate phosphorylation of the 28 kDa protein, while 12-O-tetradecanoylphor-

bol-13-acetate (TPA) was able to stimulate phosphorylation of the two 66 and the 36 kDa proteins. TPA is a direct activator of the phospholipid-Ca^{2+}-dependent protein kinase also known as protein kinase C (PKC). In addition, chelation of intracellular calcium blocked phosphorylation of the 36 and 28 kDa proteins, as well as the calcium signal and parietal cell secretory activity as measured by aminopyrine uptake[44].

In vitro phosphorylation of gastric mucosal membranes prepared from homogenized rat fundic mucosa also suggests that Ca^{+} plays a role in gastric protein phosphorylation[79]. Phosphorylation of an 88 kDa membrane protein was dependent on Ca^{2+} but was not affected by cAMP, cyclic GMP (cGMP), or CAPK inhibitor. This activity may represent a Ca^{2+}-calmodulin-dependent kinase, since trifluoperazine, an inactivator of calmodulin, blocked this Ca^{2+}-dependent phosphorylation.

Several other protein kinase activities have been described in gastric mucosa. In rabbit gastric glands, a Zn^{2+}-dependent kinase activity phosphorylates a 33 kDa protein[80]. However, because this kinase activity was observed in homogenates made from a mixed population of cells, it may actually not be specific to the parietal cell. In homogenates made from parietal cells, several protein kinase activities have been observed, including a Ca^{2+}-phospholipid-dependent kinase, two casein kinases that may correspond to casein kinase I and II[71], and a calmodulin-dependent kinase thought to be calmodulin kinase III[81].

CHAPTER TWO

THE TYPE II CAMP-DEPENDENT PROTEIN KINASE

As discussed in the previous section, earlier work on the parietal cell has indicated that protein kinases may play a role in secretagogue-stimulated acid secretion. More specifically, the cAMP-dependent protein kinase (CAPK) is thought to mediate histamine-stimulated acid secretion. The research presented here focuses on the role of the regulatory subunit of the Type II CAPK (RII). In order to place this work in context, the literature on the Type II CAPK and RII is reviewed in this section.

PROTEIN KINASES-BACKGROUND AND HISTORY

Protein kinases catalyze the transfer of phosphate groups to substrate proteins. Protein phosphorylation has been implicated in the regulation of many cell processes, including synaptic transmission[82], protein synthesis[83], growth[84], secretion[85], smooth muscle contraction and relaxation[86], DNA synthesis[87], and glycogen metabolism[88]. Although almost a hundred different protein kinases have been described, they are all thought to be related, with highly conserved catalytic domains[89]. They can be divided into two broad categories: those that phosphorylate serine or threonine residues and those that phosphorylate tyrosine residues[89]. Many of these enzymes are activated when an extracellular effector molecule binds to a membrane receptor. This results in a change in the concentration of an intracellular second messenger that in turn activates the kinase. The kinase then phosphorylates one or more substrates. As some of these substrates may

themselves be protein kinases, the stimulus may lead to a cascade of phosphorylation reactions that ultimately produce the physiological response characteristic of the effector molecule.

Protein phosphorylation is an effective and sensitive regulator of cell function because of its reversibility. The phosphate groups added by the kinases can be removed by either phosphoprotein phosphatases or the reverse reactions of the protein kinases themselves[90]. The mechanism is also made more responsive by the fact that substrates are often phosphorylated by multiple kinases, and the kinases themselves can act on more than one substrate.

The fundamental model for protein kinase activity was provided by work done on the regulation of glycogen metabolism[88]. The enzyme phosphorylase, which catalyzes the initial step in glycogen breakdown, was found to exist in two forms: an active, phosphorylated form and an inactive, dephosphorylated form. Interconversion of the two forms was regulated by two enzymes: phosphorylase kinase and phosphorylase phosphatase. Like phosphorylase, phosphorylase kinase was also found to exist in two forms, requiring the addition of a phosphate group for activation. This implied that a second kinase existed. cAMP was also known to play a role in epinephrine- and glucagon-stimulated glycogen breakdown. The relationship of these facts was made clear when Walsh et al. isolated the cAMP-dependent protein kinase (CAPK)[91].

This work provided an elegant model for hormone-stimulated regulation of a cellular process mediated by the activation and deactivation of protein kinases. However it was also significant in that it first identified cyclic AMP-dependent kinase

(Figure 5). This enzyme was soon found to be ubiquitous, phosphorylating a wide variety of substrates in many organisms and tissues[92,93] in response to many different stimuli. Nor was it confined to a single compartment of the cell. While predominately identified in soluble fractions, it was also present in association with particulate cellular matter, particularly in brain tissue[94,95].

CYCLIC AMP-DEPENDENT PROTEIN KINASE (CAPK)

CAPK phosphorylates serine and threonine residues. The enzyme appears to recognize these amino acids when a pair of basic amino acids is found in close proximity on the amino terminal side[96]. Physiological substrates of CAPK include other enzymes involved in carbohydrate metabolism as well as troponin I, cholesterol esterase, tyrosine hydroxylase, and ATP-citrate lyase[97]. In addition the enzyme exhibits broad substrate specificity in vitro that may not be physiological, phosphorylating proteins such as histone, protamine, S-6 ribosomal protein, myelin basic protein, and casein[97]. It has been hypothesized[98] but not proven[96] that all the effects of cAMP are mediated by CAPK. At least one study appears to contradict this hypothesis. In rat parotid, inhibition of the cAMP-dependent protein kinase inhibited cAMP-dependent protein phosphorylation but did not interfere with cAMP-evoked amylase release[99].

In the absence of cAMP, CAPK is inactive. The enzyme is a tetramer, composed of two identical regulatory subunits (R) and two identical catalytic subunits (C) that bind to each other with high affinity[97]. Each R subunit has two cAMP binding sites. The two sites have differing affinities for cAMP[100]. However

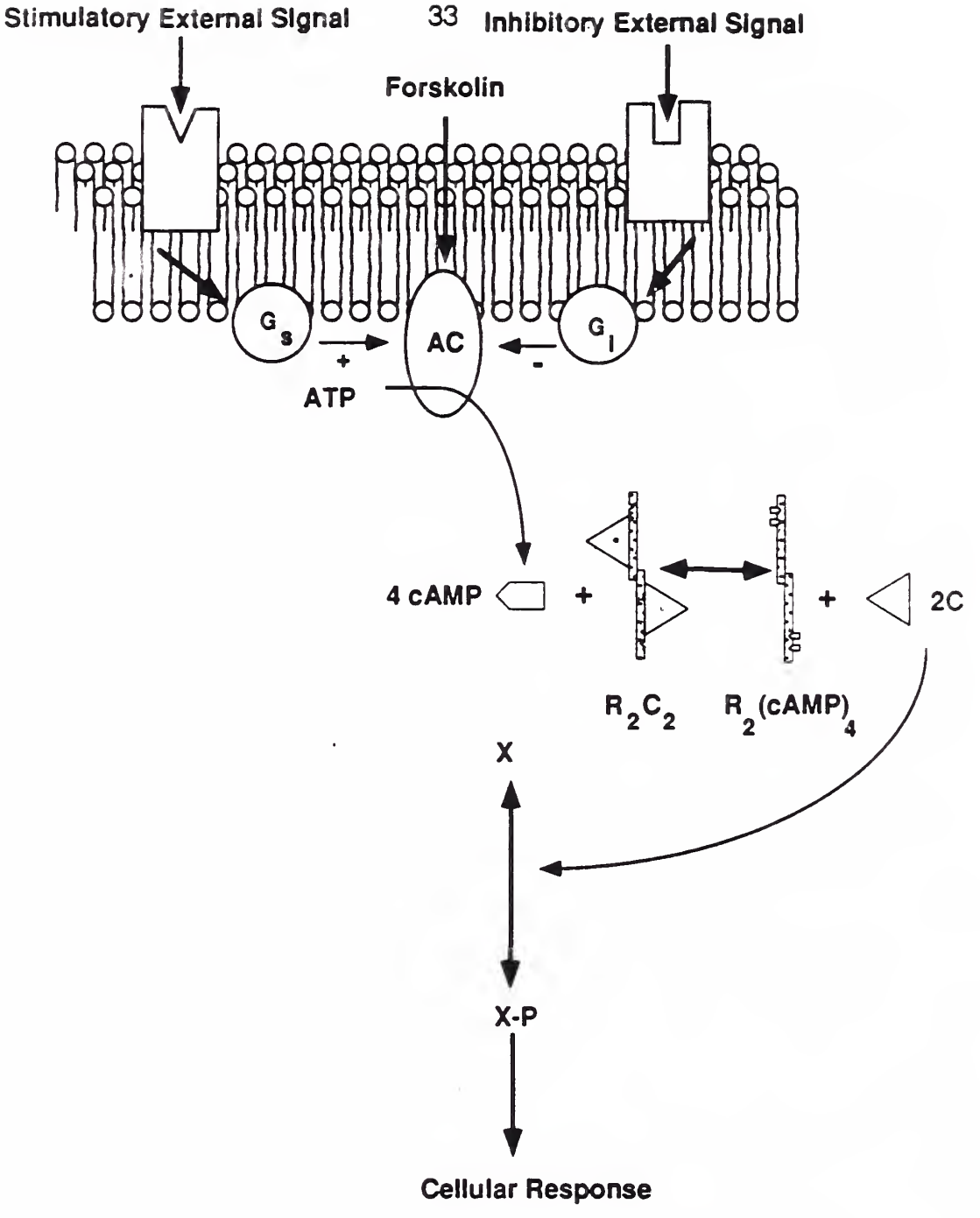

FIGURE 5: The cAMP-dependent protein kinase: mechanism of action. Stimulatory and inhibitory external signals interact with receptors on the cell surface. These interact in turn with the stimulatory and inhibitory GTP-binding proteins, G_s and G_i. G_s stimulates the enzyme adenylate cyclase (AC) to convert ATP to cAMP, whereas G_i inhibits this reaction. AC can also be directly stimulated by forskolin. The increase in intracellular cAMP activates the cAMP-dependent protein kinase by binding to the regulatory subunit (R) and releasing free catalytic subunit (C). C catalyzes the addition of a phosphate group (P) to a substrate protein (X). The activated protein (X-P) causes a cellular response.

cAMP binding to the R subunit is cooperative[94]. When cAMP binds to the R subunit, its affinity for the C subunit is diminished by a factor of 10^4, and it releases the free catalytic subunit[97]. The formula for this reaction is:

$$R_2C_2 + 4\ cAMP \rightleftharpoons R_2(cAMP)_4 + 2C \quad [97]$$

The free catalytic subunit is then active in the presence of ATP and Mg^{2+}. This activity can be inhibited by adding either a heat-stable inhibitor protein that has been purified from rabbit skeletal muscle[101] or a synthetic substrate analogue, known as CAPK pseudosubstrate[102].

Although the literature commonly refers to CAPK as though it were a single enzyme, there are in fact two isozymes[103], referred to as Type I and Type II CAPK. These were first noted to elute differently from DEAE-cellulose[103]. The two isozymes were found to have different affinities for ATP, cAMP, and the catalytic subunit[104]. While the concentration of CAPK present in a mammalian tissues is generally constant[105], the relative proportions of the two isozymes vary from tissue to tissue[93] and between species in the same tissue[106]. Subsequently it was discovered that the isozymes differed in the regulatory subunit; the catalytic subunit in each was found to be the same.

RI and RII differ in both their chemical and biological properties. While review of the literature reveals a range of sizes for each subunit, RI is generally smaller than RII. RI has been reported to have a molecular weight between 47 and 49 kDa, while RII ranges from 51 to 62 kDa. They have different patterns of immunoreactivity; antiserum raised against the Type II kinase does not react with the Type I isozyme[107]. Similarly, peptide mapping of RI and RII gave further

evidence to support the suggestion that the two forms had different amino acid sequences[108]. Amino acid analysis confirmed this suspicion[109]. Final proof was provided when the two proteins were sequenced in 1984[110,111]. RII also differs from RI in its ability to undergo "autophosphorylation" by the catalytic subunit[112]. This reaction will be more fully discussed in the next section.

The occurrence in nature of two kinds of regulatory subunit, often found within the same cell, suggests that they serve different purposes. Numerous studies done in many different systems support this hypothesis. Differential activation of the two protein kinase isozymes has been seen in rabbit corpora lutea stimulated with human chorionic gonadotropin[113], mouse pituitary tumor cells stimulated with corticotropin releasing factor[114], rat liver stimulated with glucagon[115], and histamine-stimulated rabbit parietal cells[71]. In one study, different patterns of kinase activation were seen in the same cell line when two different hormones were used to stimulate cells[116]. In cells grown from rat calvaria, parathyroid hormone stimulated Types I and II equally, whereas PGE_2 stimulated Type II CAPK almost exclusively. Site-specific cAMP analogues have also been used to determine whether cAMP binding to one of the two cAMP binding sites preferentially activates one form of the enzyme. In a human colon cancer line, the site-specific cAMP analogue 8-chloro-cAMP decreased the amount of RI and increased the amount of two species of RII[117]. Lastly, changes in the relative amounts of RI and RII have been seen in Friend erythroleukemic cells undergoing differentiation[118].

This differential activation may reflect subcellular compartmentalization of the isozymes. Data from several systems, including the rabbit parietal cell, support this

hypothesis. In rabbit parietal cells the Type II kinase is found in both soluble and particulate fractions, whereas the Type I is found only in soluble fractions[71]. Similarly, in the rat parotid RII is the dominant form in the particulate fraction, while RI is concentrated in the soluble fraction[119].

The distribution of CAPK activity has also been studied in forskolin-stimulated cells [71,114]. Forskolin activates adenylate cyclase, which catalyzes the formation of cAMP. When forskolin was used, both forms of CAPK were activated equally. These data suggest that specific hormone-receptor interactions may activate only a portion of the adenylate cyclase in the cell, increasing the local concentration of cAMP, and thus activating only that form of CAPK that is found in close proximity. However an alternative possibility is that hormones may initiate different modifications to the two proteins, enabling them to respond differently to an increase in second messengers.

Other important differences exist between the two kinds of regulatory subunit. However the data presented in this paper focus primarily on the role of RII in histamine-stimulated acid secretion in the isolated rabbit parietal cell. The properties of the Type II regulatory subunit are therefore reviewed more completely in the next section.

THE TYPE II REGULATORY SUBUNIT OF CAMP-DEPENDENT PROTEIN KINASE

The regulatory subunit of the Type II CAPK (RII) can be divided into several distinct domains. It is, like RI, a cAMP-binding protein with two cAMP binding

sites[109]. It also has at least five potential phosphorylation sites per molecule[120]. However, when isolated from bovine cardiac muscle, RII in fact contains between 1.2 and 2.7 mol phosphate/mol RII monomer[121]. RII also possesses a domain that acts to inhibit the kinase activity of the catalytic subunit and a region that interacts with another RII monomer to form a dimer[97] (Figure 6).

Autophosphorylation of RII

The tendency of RII to undergo "autophosphorylation" by the catalytic subunit of CAPK is one of its most distinctive features[112,122]. In the presence of Mg^{2+} and ATP, RII is phosphorylated at a single serine residue, incorporating 1 mole of phosphate per mole of RII subunit. This reaction demonstrates a broad pH optimum (pH 6 to 8.5) and a $K_{½}$ for ATP of 0.4 μM[123]. In contrast, the $K_{½}$ for exogenous proteins is about 12 μM[123]. Because of this high affinity, it has been assumed that in vivo this site is usually phosphorylated. Indeed, when live rats were injected with $^{32}P_i$, the majority of the RII prepared from brain, heart, and liver cytosol was found to be phosphorylated[124]. Unlike the action of the catalytic subunit on exogenous proteins, the phosphorylation of RII can take place in the undisassociated holoenzyme. This reaction is therefore unaffected by cAMP, protein kinase inhibitor (which acts on free C), or holoenzyme concentration[125]. The autophosphorylation reaction is thought to regulate the activity of Type II CAPK, since phosphorylation affects the rate of reassociation between C and RII[126]. The dephosphorylated form reassociates with C at a rate that is at least five times greater than that of phosphorylated RII (RII-P). Thus phosphorylation of RII

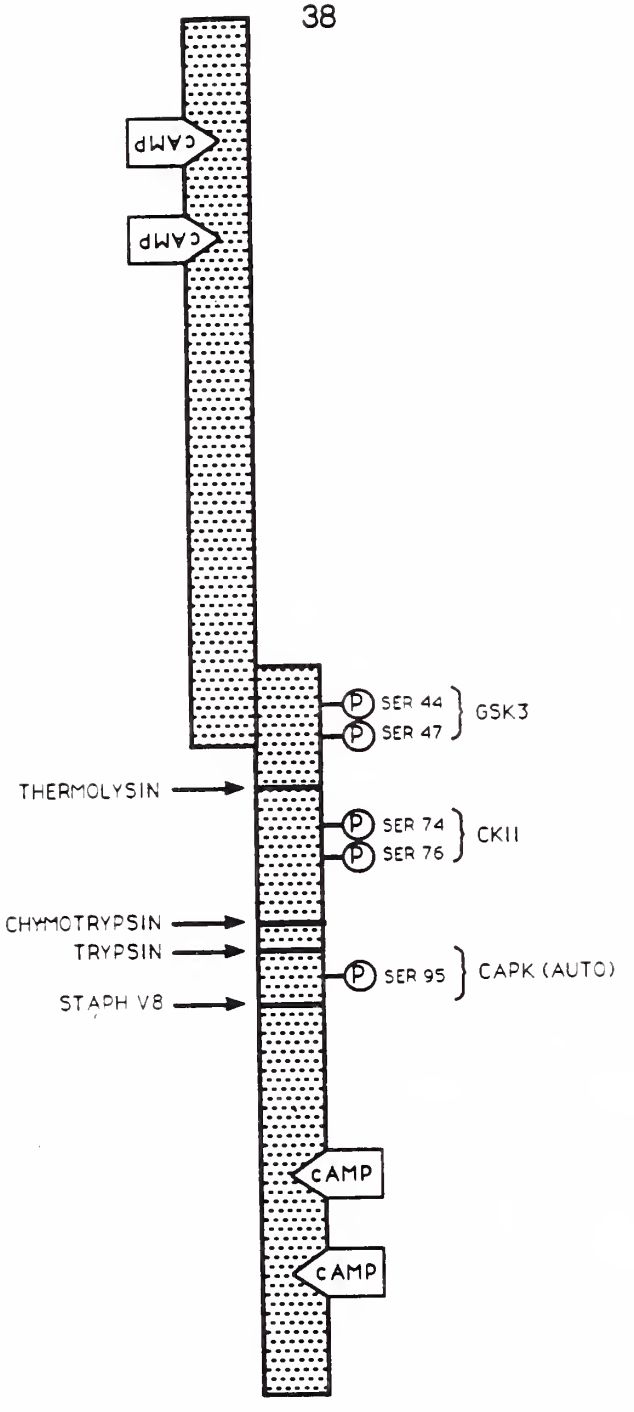

FIGURE 6: The regulatory subunit of Type II cAMP-dependent protein kinase. This diagram of the RII dimer shows regions susceptible to proteolytic digestion, potential phosphorylation sites, the two cAMP binding sites, and the dimerization domain. Arrows mark regions susceptible to different proteases. STAPH V8 - staphylococcus aureus V8 protease; SER - serine residue; GSK 3 - glycogen synthase kinase 3 phosphorylation sites; CK II - casein kinase II phosphorylation sites; CAPK(AUTO) - autophosphorylation site of cAMP-dependent protein kinase; P - phosphate.

prolongs the activity of the kinase.

There are two mechanisms by which the phosphate group can be removed from the autophosphorylated RII[123,124]. First, the phosphorylation reaction can be reversed in the presence of ADP and Mg^{2+}[123]. Unlike the forward reaction, this reaction demonstrates a pH optimum at pH 5.5. The phosphate group can also be removed by phosphatases, including a phosphatase found in bovine cardiac muscle[127].

Other phosphorylation sites on RII

The autophosphorylation reaction is not the only phosphorylation reaction associated with RII. In vitro, glycogen synthase kinase 3 (GSK 3) and casein kinase II (CK II) can phosphorylate other serine residues, distinct from the serine-95 which is autophosphorylated[128,129]. GSK 3 phosphorylates serine-44 and serine-47, while CK II phosphorylates serines-74 and 76. The CK II and autophosphorylation sites can be distinguished with phosphopeptide mapping[130]. It must be recognized that these in vitro phosphorylation activities may not be physiological. Instead these reaction may simply reflect a similarity in the amino acid sequence of the phosphorylation sites of glycogen synthase and RII, since C also phosphorylates glycogen synthase. However, RII isolated from rat soleus that had been incubated with $^{32}P_i$ is phosphorylated at the CK II site, indicating that this site is a substrate in vivo[129]. Phosphorylation at the CK II site has no known effect on the function of RII[131]. However a phosphorylated form of RII has been found to have topoisomerase activity[132], although the phosphorylation site responsible for this

activity has not been identified.

The phosphorylation state of RII has been studied in several systems. In smooth muscle from rat trachea, incubation with isoproterenol, a β-adrenergic agonist that increases the cAMP concentration, caused the amount of phosphorylated RII to decrease[133]. The direct addition of cAMP to muscle extracts in vitro also caused a decrease in phosphorylated RII[133]. A similar dephosphorylation of a 55 kDa RII was seen when cAMP was added to membranes prepared from rat liver[134]. While the phosphorylation site in rat tracheal tissue was not identified, phosphopeptide maps of the 55 kDa phosphoprotein described in rat liver membranes were identical with those of RII phosphorylated by CK II. cAMP also caused the dephosphorylation of the autophosphorylated CAPK regulatory subunit in Blastocladiella emersonii zoospores[135]. Although zoospores have only one type of R, it appears to resemble RII[135]. These cAMP-dependent reactions indicate that endogenous phosphatases are capable of dephosphorylating RII. The cAMP-dependence of these reactions may indicate that the holoenzyme must disassociate for the phosphate group to be accessible. Alternatively one or more phosphatases may be directly or indirectly activated by the presence of cAMP.

RII subtypes

RII is further distinguished from RI by the existence of a number of different subclasses. As many as six have been identified[136], and in at least one instance two different forms have been found within the same cell[137]. Like RI and RII, the RII subtypes may be under differential control[137]. The two forms that have been

most extensively studied are referred to as the brain and heart isoforms, RII-B and RII-H. They were initially distinguished from one another in these tissues by their differences in immunoreactivity[138,139]. It was also observed that autophosphorylation changes the mobility of RII-H on SDS-PAGE, whereas that of RII-B remains unchanged[140]. While the heart subclass is found in most tissues, the neural form seems to be more restricted in its distribution. It has been found in ovarian tissue[141], testis, and adrenal tissue[142] as well as brain. The two forms also seem to differ in their intracellular distribution. RII-H is predominately cytoplasmic while RII-B associates with particulate material[138]. Inspection of the sequences of neural and non-neural subtypes has shown that each possesses sites for autophosphorylation and phosphorylation by CK II[142]. However the neural type does not appear to have the consensus sequence corresponding to the glycogen synthase kinase 3 phosphorylation site[142].

Interactions with other proteins

Studies undertaken in brain first suggested that RII might play a role other than regulation of CAPK. Previously it was believed that in eukaryotic cells, all effects of cAMP were mediated by CAPK[98]. Thus it was assumed that the cAMP-binding proteins RI and RII served no other purpose than to inhibit the catalytic subunit. However in brain, RII is found to be tightly associated with microtubule-associated protein 2 (MAP 2)[143]. Neither cAMP nor catalytic subunit inhibit this binding, suggesting that it involves a domain that does not interact with cAMP or C. In brain, RII also interacts with a 75 kDa calmodulin-binding protein[144], the

calmodulin-dependent phosphatase calcineurin[169], and an unidentified 80 kDa protein[145]. RII-binding proteins are also found in heart but appear to be different from those found in brain[145].

These RII binding proteins may serve to fix and compartmentalize CAPK, bringing it in close proximity with specific substrates. Alternatively, the interaction of RII with proteins other than C may indicate that RII serves functions other than the regulation of the kinase. The latter hypothesis is consistent with evidence suggesting that in some cells free RII exists in excess of C[118]. In either case it is plausible that RII may be involved in cytoskeletal regulation. Not only does RII associate with MAP-2, a protein found in close proximity to microtubules; immunocytochemistry localized RII to microtubules, microtubule-organizing centers, and parts of the Golgi complex[146]. This association with the cytoskeleton is of particular relevance in the evaluation of the role of RII in the parietal cell, since the cytoskeleton is thought to be critically involved in the morphological transformation associated with secretion.

CHAPTER 3

MATERIALS AND METHODS

MATERIALS

New Zealand white rabbits (2-3 kg) were obtained from Camm (Wayne, NJ). Pronase (Protease E), collagenase (type I), histamine chloride, CAPK catalytic subunit, kemptide, heat stable CAPK inhibitor from rabbit muscle (Product No. P 5015), and CAPK pseudosubstrate (Product No. P3294) were purchased from Sigma Chemical Co. (St. Louis, MO). Forskolin was purchased from Calbiochem (La Jolla, CA). Cimetidine was obtained from SmithKline and French (Philadelphia, PA). [^{14}C]aminopyrine was obtained from Amersham (Arlington Heights, IL). [gamma-^{32}P]-ATP (5-10 Ci/mmol) was purchased from New England Nuclear (Boston, MA). [^{125}I]-goat anti-mouse IgG and 8-azido-[^{32}P]-cAMP were purchased from ICN (Costa Mesa, CA). Calmodulin was produced from bovine brain by Dr. James Goldenring (Yale University, New Haven, CT) using the method of DeLorenzo and Goldenring[147]. Molecular weight markers, carbamylated isoelectric focusing standards, and Protein A beads were products of Pharmacia (Piscataway, NJ). Immobilon P was purchased from Millipore (Bedford, MA). Cyclic AMP assay kits were bought from Diagnostic Products Corporation (Torrance, CA). Okadaic acid was a gift from Dr. Harold Rasmussen (Yale University, New Haven, CT). A monoclonal antibody against the gastric H^+/K^+-ATPase was the gift of Dr. Adam Smolka (University of South Carolina, Charleston, SC). The monoclonal antibodies 6H11, 2E11, and 4A5 against the 80 kDa parietal cell apical membrane protein were the gift of Dr. John Forte (University of California, Berkeley, CA).

Polyclonal antibodies against rat heart and rat brain RII were the gift of Dr. Suzanne Lohmann and Dr. Ulrich Walter (University of Würzburg, West Germany) and Dr. Pietro DeCamilli (Yale University, New Haven, CT). All other reagents were from standard suppliers and were of the highest purity available.

PREPARATION OF CELLS

Parietal cells were prepared by a modification of techniques described for isolation of gastric glands[25] and parietal cells[42,148,149]. After high pressure aorto-celiac perfusion, fundic mucosa was bluntly separated from submucosa. Mucosal fragments were digested for 20 min at 37°C under 100% O_2 with 0.5 mg/ml pronase in 50 ml of solution 1 (130 mM NaCl; 12 mM $NaHCO_3$; 3 mM NaH_2PO_4; 3 mM Na_2HPO_4; 3 mM K_2HPO_4; 2 mM $MgSO_4$; 1 mM $CaCl_2$; 0.03 mM bovine serum albumin (fraction V) (BSA); 11 mM dextrose; and phenol red, 10mg/l, pH 7.4). After collagenase digestion the tissue was washed three times by pelleting and resuspension in solution 2 (132.4 mM NaCl; 5.4 mM KCl; 5 mM Na_2HPO_4; 1 mM NaH_2PO_4; 1.2 mM $MgSO_4$; 1 mM $CaCl_2$; 0.5 mM dithiothreitol (DTT); 1 mM pyruvate; 10 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) pH 7.4; 0.06 mM BSA; 10 mM dextrose; and phenol red 10mg/l). After washing, the tissue was resuspended in 50 ml of solution 1 containing collagenase 0.8 mg/ml and allowed to incubate for 45 min at 37°C under 100% O_2. DNAse 0.5 mg/ml was added for the last ten minutes. Dispersed cells were then washed three times in solution 2, filtered through 30 μm nylon mesh and resuspended in 10 ml of solution 2.

The cells were then fractionated on discontinuous Nycodenz gradients. These were prepared in the following manner: isotonic sterile Nycodenz solution containing 0.06 mM BSA was diluted 1:1 (density 1.075 g/ml) and 1:2 (density 1.050 g/ml) with diluent solution (5 mM Tris-HCl, pH 7.4; 3 mM KCl; 0.3 mM CaNa-EDTA; 128.3 mM NaCl; and 0.06 mM BSA). Gradients were prepared by placing 5 ml of the 1:1 solution below 8 ml of the 1:2 solution. Two ml of the cell suspension were layered on top of each gradient, which was then centrifuged at 200 x g for 10 min at 10°C. Enriched parietal cells (purity 85 ± 5%) were removed from the interface between the 1.075 g/ml and the 1.050 g/ml solutions. These cells were washed three times by pelleting and resuspension in solution 2. Solution 2 was then used to bring the cells to the desired concentration. All steps used a Beckman J6M refrigerated centrifuge.

PREPARATION OF SUBCELLULAR FRACTIONS

Purified parietal cells were resuspended in solution 2 to a concentration of 5 x 10^6 cells/ml. After a 10 min recovery period at 37°C under 100% O_2, cells were incubated for 45 min more with either 100 μM cimetidine (unstimulated cells) or 100 μM histamine plus 10 μM forskolin (maximally stimulated cells). Subcellular fractions were then prepared using a modification of the method developed for gastric glands by Urushidani et al.[75].

Cells were pelleted at 50 x g for 5 min at 4°C. Homogenization buffer (110 mM mannitol; 10 mM sucrose; 150 mM NaCl; 1 mM $MgCl_2$; 5 mM benzamidine; 1 mM PMSF; 1 μM aprotonin; and 1 μM leupeptin) was added to the cell pellets

(0.5 ml/million cells), which were then homogenized on ice with 15 strokes of a Potter-Evelyn homogenizer at 900 rpm. Subcellular fractions were then prepared by differential centrifugation (Figure 7). Cell homogenates were first centrifuged for 5 min at 40 x g to make the P_0 pellet. The supernatant from this pellet was then centrifuged for 10 min at 4000 x g to make the P_1 pellet. The supernatatant was again centrifuged, this time for 10 min at 14,500 x g to make the P_2 pellet. Finally, the remaining supernatant was spun once more at 48,500 x g for 60 min to produce the P_3 pellet and the final, high-speed S_3 supernatant. All centrifugation was conducted at 4°C. Pellets were washed with homogenization buffer and resuspended in resuspension buffer (200 mM sucrose, 100 mM NaCl, and 0.5 mM EDTA). Low speed centrifugation was undertaken in a Beckman J6M refrigerated centrifuge, while the final high-speed spin required a Sorvall OTD-2 ultracentrifuge.

CHARACTERIZATION OF SUBFRACTIONS

Immunoblotting

Western blotting was used to determine the distribution of the H^+/K^+-ATPase and the 80 kDa apical membrane protein[77]. Samples of both stimulated and unstimulated subcellular fractions were resolved on 10% SDS-PAGE minigels and transferred to Immobilon overnight at 50 milliamps[150]. Blots were blocked with Blotto (5% non-fat dry milk; 0.1% Triton X-100; 25 mM Tris HCl, pH 7.5; and 150 mM NaCl) for two hours. The blots were then incubated overnight with either a monoclonal antibody directed against the H^+/K^+-ATPase (1:150 dilution) or a mixture of three monoclonal antibodies against the 80 kDa protein (1:100 dilution).

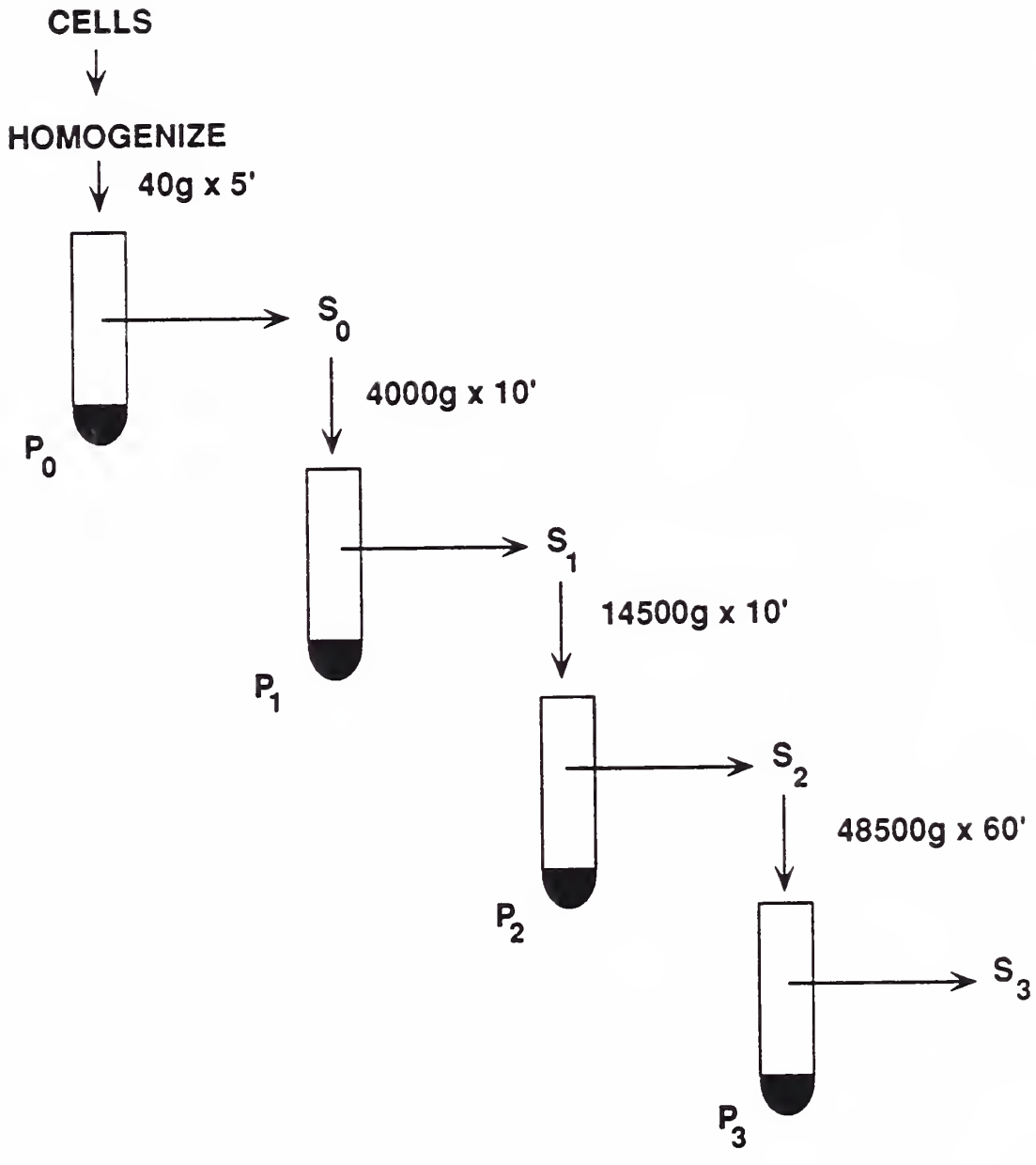

FIGURE 7: Protocol for preparation of subcellular fractions.
Differential centrifugation was used to prepare subcellular fractions from homogenized parietal cells. For a more detailed description, see text. (P - pellet; S - supernatant).

After this incubation, blots were washed 3 times in Blotto, then incubated with [^{125}I]-goat anti-mouse IgG for 2 hours. The blots were then washed 3 more times with Blotto before drying.

Dried blots were exposed to x-ray film to locate the bands of interest. The data were quantified by cutting out the labeled bands and counting them in a gamma counter. Data were then expressed as cpm/mg total protein in the original sample.

Cyclic AMP-dependent protein kinase activity

CAPK activity was determined using kemptide[102], a synthetic substrate peptide for CAPK. Prior to assay, membrane fractions were solubilized in 0.5% Triton X-100 for 30 min on ice. In the final reaction mixture, the Triton was diluted to 0.04%. Cytosolic fractions were used without any further preparation. The standard 50 μl reaction mixture contained 5 μg kemptide; 25-50 μg protein sample; 50 mM HEPES, pH 7.0; 10 mM $MgCl_2$; and 100 μM [gamma-^{32}P]-ATP (100-400 cpm/pmol). Reactions were also performed in the absence of kemptide. Another set of reactions was performed in the presence of 10 μM cAMP. All reactions were allowed to proceed at 35°C for 30 min. After 30 min, four 10 μl samples were withdrawn from each reaction tube and spotted on 2 x 1 cm pieces of Whatman P81 paper. This is a basic paper that binds the positively charged kemptide. Free ATP was then removed by washing the pieces of paper 4 times for 10 min each in 50 mM phosphoric acid. After an acetone rinse, papers were then dried and Cherenkov radiation counted. The phosphorylation of kemptide was determined

by subtracting the amount of bound radioactivity seen in the presence of sample alone from that seen in the presence of kemptide.

Other parameters

To quantify the presence of nuclear material in subcellular fractions, DNA content was determined using the method of West et al.[151]. Fractions were also evaluated for the presence of mitochondria using cytochrome C oxidase activity as a marker. Cytochrome C oxidase activity was determined by the method of Peters et al.[152]. Protein content of each fraction was determined by the method of Bradford[153], using BSA as a standard.

IN VITRO PHOSPHORYLATION

In vitro phosphorylation of endogenous proteins in subcellular fractions was assayed using 10 μl of protein sample in a reaction volume of 100 μl. The standard reaction mixture contained 50 mM HEPES (pH 7.0), 10 mM $MgCl_2$, 0.5 mg/ml DTT, and 10 μM [gamma-^{32}P]-ATP. Reactions were carried out in both the presence and absence of 10 μM cAMP. Reactions were initiated by the addition of [gamma-^{32}P]-ATP and were allowed to proceed for 60 sec at 35°C. Reactions were stopped by the addition of 50 μl of 3% SDS stop solution followed by boiling for 5 min. After the samples cooled, 20 μl of 2-mercaptoethanol was added to each sample prior to loading for SDS-PAGE.

The standard reaction mixture was varied by changing the concentration of Mg^{2+} (0.1 mM to 30 mM), ATP (0.1 μM to 50 μM), and cAMP (0.1 nM to 10 μM).

A time course of the standard reaction was performed by stopping the reactions at times from 5 sec to 5 min. To determine whether the kinase could also use GTP as a substrate, in some experiments GTP (0.1 μM to 50 μM) was substituted for ATP. To determine whether cAMP had a unique effect on the phosphorylation reaction, cyclic GMP (0.01 μM to 10 μM) was substituted for cAMP in some experiments. The pH of the reaction was varied by using MES and HEPES buffers ranging from pH 5.5 to pH 8.0. Reactions were also carried out in the presence of the heat stable CAPK inhibitor (1 μg/ml), CAPK pseudosubstrate (0.2 units/μl), CAPK catalytic subunit (0.1 units/μl), calmodulin (0.01 mg/ml), $CaCl_2$ (1 mM), $MnCl_2$ (10 mM), $ZnSO_4$ (10 mM), or EDTA (2 mM; exogenous $MgCl_2$ was not added to the reaction containing EDTA).

To evaluate the presence of a phosphatase in the P_1, standard <u>in vitro</u> phosphorylation reactions were stopped after 60 sec with 10 mM EDTA. cAMP was immediately added and the reaction was then stopped at various time points from 0 sec to 4 min with 50 μl of 3% SDS stop buffer. The dephosphorylation seen in the presence of 10 μM cAMP was evaluated by observing the effect of known phosphatase inhibitors including $MnCl_2$ (10 mM), $ZnSO_4$ (10 mM), NaF (50 mM), pyrophosphate (10 mM), and okadaic acid (0.01 μM to 10 μM). At the end of standard 1 min phosphorylation reactions, 10 mM EDTA was added to stop further phosphorylation. Potential phosphatase inhibitors were then added to the reaction mixture with 10 μM cAMP. Reactions were allowed to run for 3 min more before being stopped with 1% SDS.

PHOSPHOAMINO ACID ANALYSIS

Phosphoamino acid analysis was performed on phosphoproteins separated on either one- or two-dimensional gels using a previously described modification of the method of Hunter and Sefton[154,155]. This method allows separation of all three stable phosphoamino acid species: phosphoserine, phosphothreonine, and phosphotyrosine.

8-AZIDO-cAMP BINDING STUDIES

Subcellular fractions were assayed for cAMP-binding proteins using 8-azido-[^{32}P]-cAMP, a photoaffinity probe which binds competitively at cAMP-binding sites. Upon excitation with ultraviolet light, this compound generates a free radical that inserts into a carbonyl bond, thus binding it irreversibly to the substrate protein.

Reaction mixtures (90 μl) containing 50 mM HEPES, pH 7.0; 0.1 mM IBMX; 10 mM $MgCl_2$; and 50-100 μg of the protein sample were prepared in a microtiter plate and placed on ice. In semi-darkness 10 μl of 8-azido-[^{32}P]-cAMP was added to each reaction. Control reactions contained in addition 100 μM nonradioactive cAMP. After mixing, the microtiter plate was wrapped in foil, placed on ice, and incubated on a rocker platform for 30 min. The plate was then unwrapped and exposed to a high power UV light (XX15, Ultra-Violet Products Inc.) at 6 inches for 5 min. Reactions were then stopped by the addition of 3% SDS stop solution (1% SDS final concentration) or isoelectric focusing stop solution (see Methods: Two-dimensional gel electrophoresis). This allowed samples to be resolved by one- or two- dimensional gel electrophoresis.

IMMUNOPRECIPITATION

Standard 100 μl phosphorylated reaction mixtures were used. After being stopped by the addition of SDS to a final concentration of 1% and boiling, samples were diluted 1:9 with RIPA[156] buffer (150 mM NaCl, 10 mM Tris HCl, pH 7.4, 10 mM sodium pyrophosphate, 20 mM NaF, 1 mM EDTA, 1% sodium deoxycholate, and 1% Triton X-100) with 0.5 mg/ml BSA and 5 mM benzamidine. Polyclonal antisera against rat heart RII and rat brain RII were used to immunoprecipitate these samples. Five μl of undiluted antiserum, 5 μl of non-immune rabbit serum, or 5 μl of RIPA were then added to each sample. Reaction mixtures were then incubated overnight at 4°C with constant agitation. Immune complexes were immunoprecipitated by incubation for 2 hours with 3 to 5 mg of pre-swollen Protein A-sepharose beads. The beads were pelleted for 5 min at 1000 x g, then washed three times in RIPA with 0.5 mg/ml BSA and 0.1% SDS, followed by two washes with RIPA alone. The washed beads were boiled for 5 min in 100 μl of 1% SDS stop solution to remove the adherent proteins. The beads were then pelleted and the supernatants collected for SDS-PAGE resolution.

WHOLE CELL STUDIES

Measurement of acid secretion

Acid secretion was assessed by the semi-quantitative uptake of [^{14}C]amino-pyrine (AP) using a modification of the method of Berglindh[157,28]. A parietal cell suspension containing 1.5x10^{6}cells/ml in solution 2 was pre-incubated with 2.12 nmol/ml [^{14}C]AP (specific activity 4.366 Bq/nmol) for 30 min at 37°C under 100%

O_2. Following pre-incubation, 100 μl aliquots (150,000) cells were added to 96 well microtiter plates with integral durapore (Millititer SV) membrane filters (5.0 μm pore size).

Cells were then incubated with okadaic acid (concentration 0.01 μM to 3 μM), the CAPK inhibitor H-8 (concentration 0.01 μM to 30 μM), or control (an equal volume of solution 2). Each condition was performed in triplicate. The plates were then placed in an oxygenation chamber and oxygenated at 37 °C for 30 minutes[158]. At the end of this time period histamine (10 μM) was added. In some okadaic acid experiments, a combination of histamine (100 μM) and forskolin (10 μM) was used instead. Cells were then incubated for an additional 45 min in the presence of 100% O_2 at 37 °C. At the end of this incubation period cells were rapidly separated from incubation media by vacuum filtration. The membrane filters containing the cells were dried, punched into scintillation vials, and counted in a liquid scintillation counter. Non-specific binding of [^{14}C]AP to membrane filters was measured in wells containing an equal amount of isotope but no cells. This non-specific binding was subtracted from binding measured in the presence of cells. Data were then expressed as a percentage of maximal [^{14}C]AP uptake.

Measurement of cAMP concentration

Cyclic AMP was assayed using a commercial assay kit (Diagnostic Products Corp.). Parietal cells were incubated in the same manner used for the secretion assay. Each condition was performed in duplicate. After vacuum filtration, the membrane filters containing the cells were punched into tubes containing 0.5 ml

ice-cold 3% perchloric acid, vortexed for 20 sec, and stored at -70°C until assay. Immediately prior to assay samples were thawed and stored on ice before adding 100 μl of cold 30% $KHCO_3$, bringing the pH to 6.0. The samples were centrifuged and 100 μl of supernatant was assayed for cAMP by competitive protein binding assay[158]. Results were expressed as pmol/10^6 cells.

GEL ELECTROPHORESIS

One-dimensional SDS-PAGE

Proteins were resolved using a modification of the method of Laemmli[159]. Discontinuous sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 10% separating gels with 4% stacking gels cast on Gelbond PAG film. After electrophoresis, the gels were stained with Coomassie blue, dried in a forced air oven at 60°C, and exposed to x-ray film (Kodak XAR-5) at -70°C for 8 to 120 hours. Molecular weights were determined using standard protein molecular weight markers.

Two methods were used to quantify protein phosphorylation. In some cases, a Biorad densitometer was used to measure the optical density of phosphorylated bands detected by autoradiography. Alternatively, ^{32}P incorporation was determined by excising bands from dry gels. The radioactivity of the band was then measured using a liquid scintillation counter. In each method, data were expressed as a percentage of maximal phosphorylation.

Two-dimensional gel electrophoresis

Proteins were separated on two-dimensional isofocusing/SDS-PAGE using a modification of the method of O'Farrell[160]. Samples were prepared by adding an equal volume of isoelectric stop buffer (4% Triton X-100; 4% 3-10 ampholines; 10% 2-mercaptoethanol; 9.5 M urea). Isoelectric focusing in the first dimension was accomplished in polyacrylamide tube gels using 3 to 10 ampholines (4%). In the second dimension, proteins were separated by SDS-PAGE, as described above. After electrophoresis, the gels were handled in the manner described for one-dimensional gels. Isoelectric points were determined using carbamylated standards.

STATISTICAL METHODS

Measurements of [^{14}C]aminopyrine accumulation and cAMP levels were compared to controls using a t-test for paired samples.

CHAPTER 4

RESULTS

Previous studies using isolated rabbit parietal cells have shown that histamine stimulates the accumulation of [^{14}C]aminopyrine, a marker for parietal cell acid secretion[157] (Figure 8). Histamine stimulation also results in an increase in the intracellular concentration of the second messenger cAMP[157] (Figure 8). The only known function of cAMP is activation of the cAMP-dependent protein kinase[98], which phosphorylates a wide variety of substrate proteins[97]. Several groups have identified a number of histamine-stimulated phosphoproteins in rabbit parietal cells and gastric glands[72,73,74,75]. We hypothesized that histamine stimulation might change the phosphorylation state of proteins found in actively secreting parietal cells. If such proteins were found, they would be likely intermediaries in histamine-stimulated acid secretion. By preparing subcellular fractions from homogenized cells we hoped to be able to localize phosphorylation changes to specific intracellular compartments.

Subcellular fractions prepared from resting and stimulated parietal cells were therefore characterized. These fractions were then assayed for kinase activity. This survey revealed that the apical membrane-rich fraction in stimulated parietal cells possesses both kinase and phosphatase activities that act on a 52 kDa protein substrate. The protein was identified as the regulatory subunit of Type II CAPK (RII). Whole cell secretion studies were then undertaken using a phosphatase inhibitor and an inhibitor of CAPK, to determine whether the kinase or a phosphatase played the key role in parietal cell acid secretion.

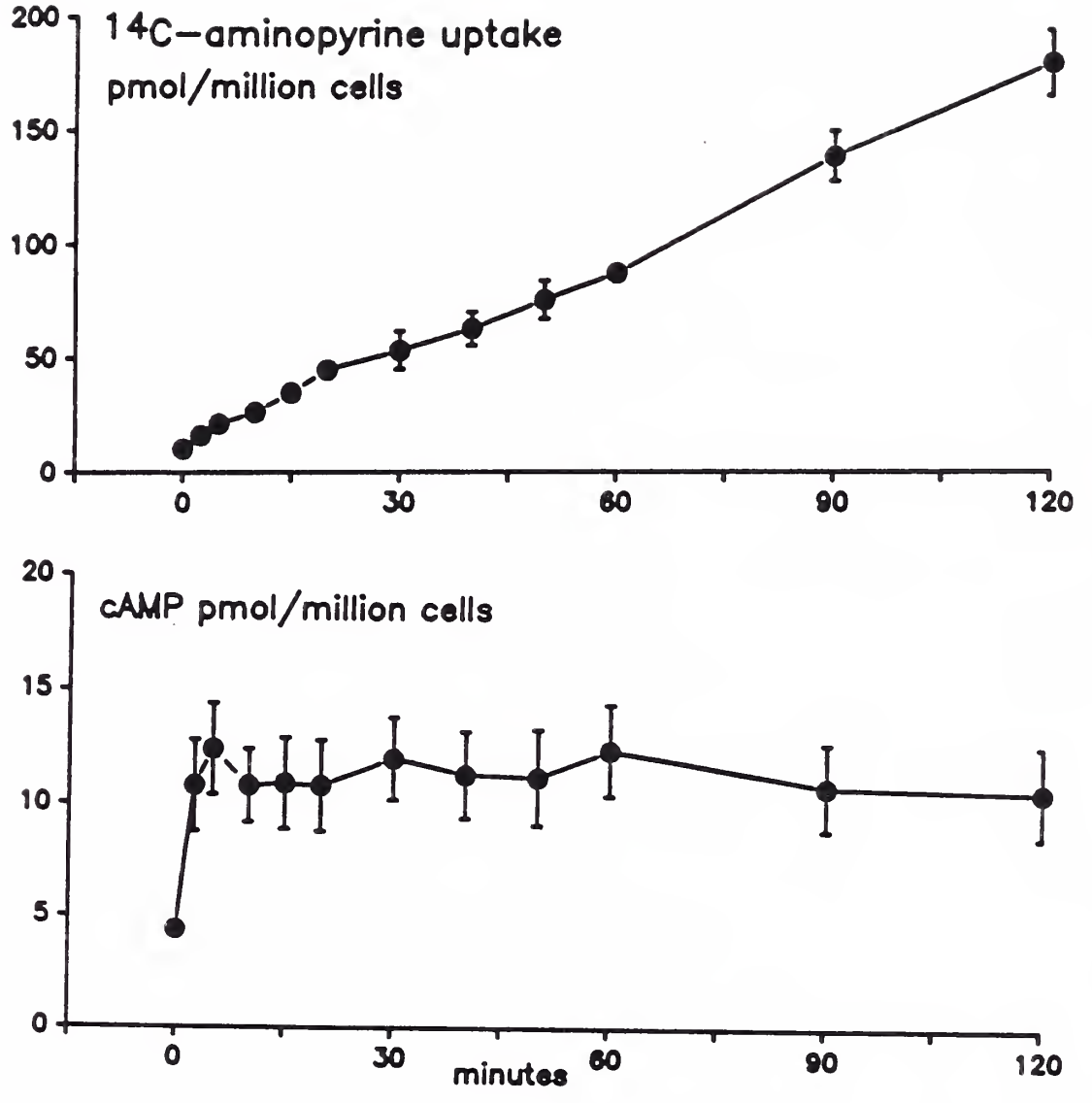

FIGURE 8: Effect of histamine stimulation of isolated rabbit parietal cells on [^{14}C]aminopyrine uptake and cellular cAMP content.
Isolated rabbit parietal cells were incubated with histamine (10 μM). [^{14}C] aminopyrine accumulation and cellular cAMP content were measured as described in Chapter 3. Top panel: time course of [^{14}C]aminopyrine uptake. Bottom panel: time course of cellular cAMP content.

CHARACTERIZATION OF SUBFRACTIONS

When Urushidani and Forte characterized subfractions prepared from rabbit gastric glands, they used K^+-p-nitrophenyl phosphatase as a marker for H^+/K^+-ATPase activity[75]. They reported that in resting glands, the bulk of the activity was present in the microsomal P_3 fraction. However in histamine-stimulated glands, the activity in P_3 decreased by 50%. This decrease was accompanied by a striking increase in activity in the apical membrane-rich P_1 fraction [75]. This data supported the hypothesis that in the stimulated cell, the H^+/K^+-ATPase moves from the lighter tubulovesicles to the heavier apical membrane. The same group has also described an 80 kDa actin-binding protein that additionally serves as a marker for the apical membrane[77]. Histamine stimulation changed the phosphorylation state of this protein without altering its subcellular distribution.

In our preparation, made from isolated rabbit parietal cells rather than glands, we used a monoclonal antibody against the H^+/K^+-ATPase to localize the proton pump and characterize the subcellular fractions. In agreement with previous studies, we found that in histamine-stimulated cells the H^+/K^+-ATPase moved from the microsomal P_3 fraction to the heavier P_0 and P_1 (Figure 9). These fractions, but particularly P_1,, in both resting and stimulated cells, also showed enriched immunoreactivity with a monoclonal antibody against the 80 kDa actin-binding protein described above (Table III). The presence of these markers in the P_1 subcellular fraction is consistent with the conclusion that the P_1 fraction was enriched with apical membrane. The P_0 fraction exhibited somewhat similar characteristics, but had the highest DNA content (Table III), indicating the additional

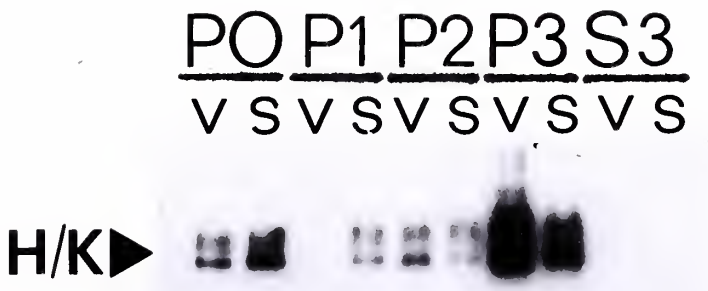

FIGURE 9: Distribution of the H^+/K^+-ATPase in subcellular fractions prepared from parietal cell homogenates.
Western blot analysis was used to study the distribution of the H^+/K^+-ATPase in subcellular fractions from maximally stimulated and unstimulated parietal cells. SDS-PAGE was used to resolve proteins from all subcellular fractions from both unstimulated (V) and maximally stimulated (S) cells (15 μg of protein per sample). Western blot analysis was then performed as described in Chapter 3, using a monoclonal antibody against the H^+/K^+-ATPase. P0, P1, P2, P3 - membrane fractions; S3 - cytosolic fraction; V - unstimulated; S - maximally stimulated; H/K - H^+/K^+-ATPase.

	VP_0	SP_0	VP_1	SP_1	VP_2	SP_2	VP_3	SP_3	VS_3	SS_3
DNA (μg/mg protein)	115	128	52	38	11	11	15	13	34	21
Cytochrome C Oxidase (A_{545} units/mg protein)	140	130	180	165	75	42	3	4	0	0
80 kDa immunoreactivity (cpm/mg protein)	2000	2100	6000	5600	1000	900	150	200	0	0
cAMP-dependent kinase (pmol/mg prot/min)	2.3	3.3	0.8	1.5	6.2	12.5	24.3	17.0	713	280

TABLE III: Characterization of subcellular fractions.
All fractions in both stimulated and unstimulated cells were assayed for DNA content, cytochrome C oxidase activity, 80 kDa protein immunoreactivity, and cAMP-dependent protein kinase activity. Unstimulated cells were treated with cimetidine (100 μM). Stimulated cells were treated with histamine (100 μM) and forskolin (10 μM). For materials and methods see Chapter 3. V - unstimulated; S - stimulated; P_o, P_1, P_2, and P_3 - membrane fractions; S_3 - cytosolic fraction.

presence of nuclear material. The P_1 fraction was slightly enriched in DNA. Cytochrome C oxidase activity, a mitochondrial marker, was enriched in the P_0 and P_1 fractions (Table III). From these data it was concluded that the P_1 fraction is the apical membrane-rich fraction, although it also contains some nuclear and mitochondrial contaminants.

IN VITRO PROTEIN PHOSPHORYLATION IN SUBCELLULAR FRACTIONS

Endogenous phosphorylation was assayed in all subcellular fractions prepared from both resting and stimulated parietal cells (Figure 10) in the presence and absence of cAMP. In the presence of 10 mM Mg^{2+} and 10 μM ATP, many proteins were phosphorylated in all fractions. However the most prominent and consistent findings were observed in the P_1 apical membrane fraction and the S_3 cytosolic fraction (Figure 11).

In the stimulated P_1 a protein with the molecular weight of 52 kDa (pp52) was phosphorylated in the absence of cAMP. The time course of this activity was extremely rapid, reaching maximal phosphorylation within 45 sec at 35°C (Figure 12). The phosphorylation of pp52 was dependent on the concentration of Mg^{2+} (Figure 13) and could be eliminated entirely by EDTA. The activity was also dependent on the concentration of ATP (Figure 14), with a $K_{½}$ for ATP of approximately 3 μM. Although the kinase could use GTP as a substrate ($K_{½}$ 10 μM) (Figure 14), it exhibited a preference for ATP. The kinase did not exhibit a pH optimum; phosphorylation took place over a pH range from 5.5 to 8.0. Phosphorylation was not affected by the presence of the heat-stable CAPK inhibitor, CAPK

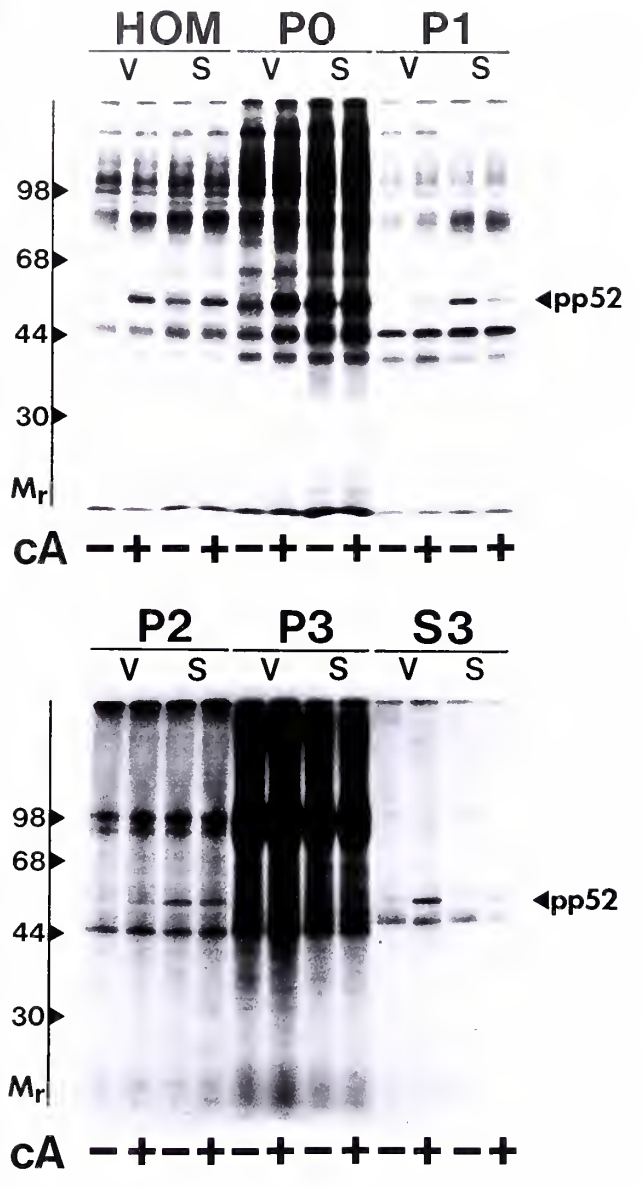

FIGURE 10: Autoradiograph showing endogenous phosphorylation in all subcellular fractions.
Standard 1 min in vitro phosphorylation reactions were performed with and without 10 μM cAMP (+/- cA) using protein samples from homogenate (HOM) and subcellular fractions P_o through S_3. Unstimulated (V) cells were treated with cimetidine 100 μM. Maximally stimulated (S) cells were treated with histamine (100 μM) and forskolin (10μM). Samples were resolved using SDS-PAGE and autoradiography. Molecular weight standards are indicated on the left. Arrows show the position of the 52 kDa phosphoprotein (pp52).

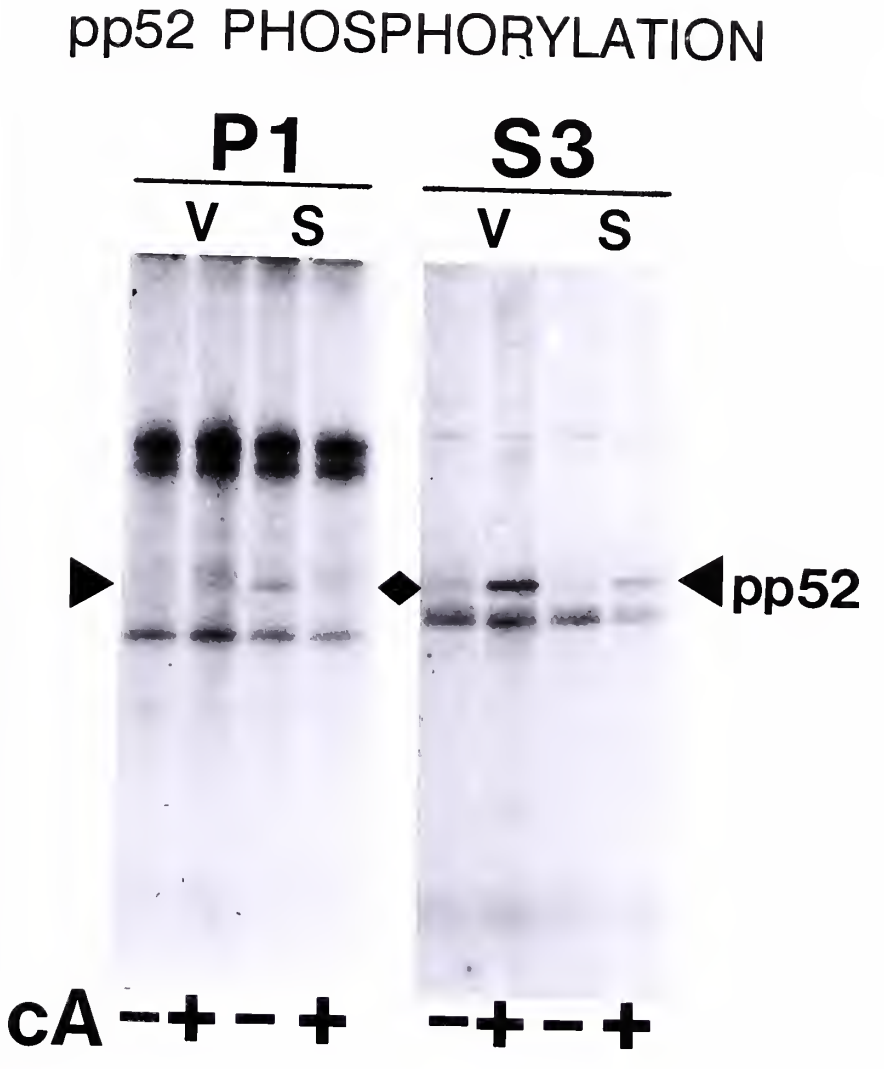

FIGURE 11: Autoradiograph showing endogenous phosphorylation in the apical membrane fraction and the cytosolic fraction.
Standard 1 min in vitro phosphorylation reactions were performed with and without 10 μM cAMP (+/- cA) using protein samples from the P_1 membrane fraction and the cytosolic S_3 fraction (S3). Unstimulated (V) cells were treated with cimetidine (100 μM). Maximally stimulated (S) cells were treated with histamine (100 μM) and forskolin (10 μM). Samples were resolved using SDS-PAGE and autoradiography. Arrows indicate the position of the 52 kDa phosphoprotein.

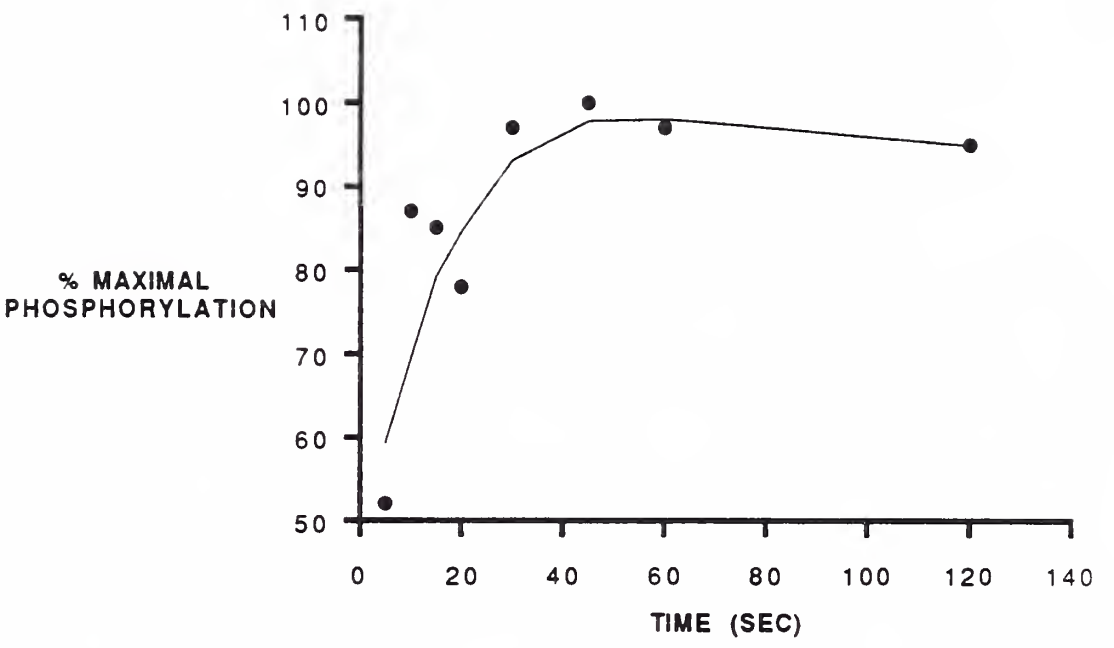

FIGURE 12: Time course of pp52 phosphorylation.
Standard in vitro phosphorylation reactions were performed using protein samples from the maximally stimulated P_1 membrane fraction. Reactions were stopped at time points from 5 sec to 300 sec. Samples were resolved by SDS-PAGE and autoradiography, and phosphorylation of pp52 was quantified as described in Chapter 3. Phosphorylation was maximal by 120 sec; therefore time points beyond 120 sec are not shown.

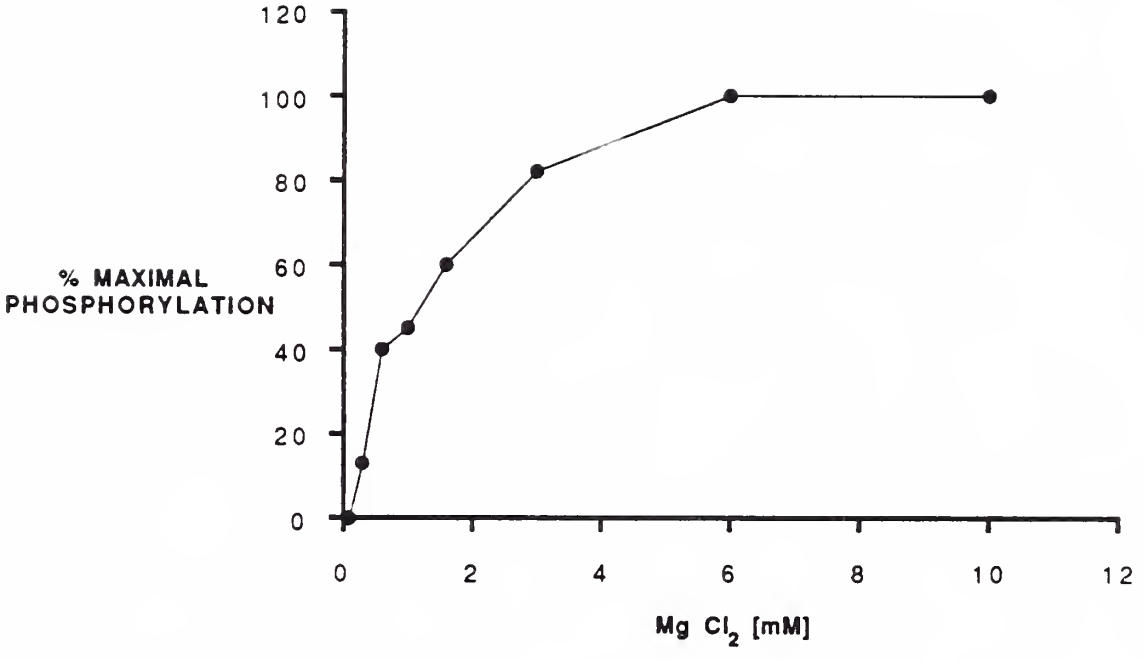

FIGURE 13: Magnesium dependence of pp52 phosphorylation.
Standard 1 min in vitro phosphorylation assays were performed using protein samples from the maximally stimulated P_1 membrane fraction. However the concentration of exogenous Mg^{2+} was varied from 0.1 mM to 30 mM. Samples were resolved with SDS-PAGE and autoradiography, and the phosphorylation of pp52 was quantified as described in Chapter 3. Phosphorylation is expressed here as percent maximal phosphorylation. Phosphorylation was maximal at 10 mM; therefore values for higher Mg^{2+} concentrations are not shown.

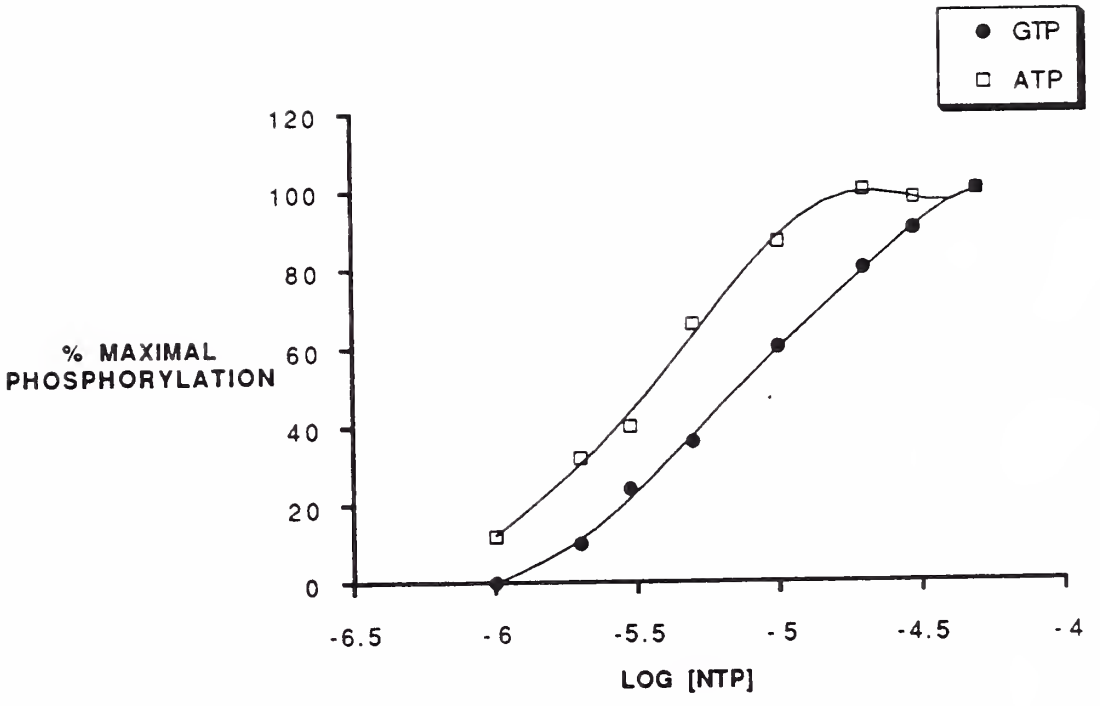

FIGURE 14: ATP and GTP dependence of pp52 phosphorylation.
Standard 1 min. in vitro phosphorylation reactions were performed using protein samples from the maximally stimulated P_1 membrane fraction. Either [gamma-^{32}P]-ATP or [gamma-^{32}P]-GTP was used as a phosphate donor with concentrations ranging from 0.1 μM to 50 μM. Samples were resolved using 1-D SDS-PAGE and autoradiography, and phosphorylation of pp52 was quantified as described in Chapter 3. Phosphorylation is expressed here as percent maximal phosphorylation. Closed circles represent the phosphorylation of pp52 in reactions using GTP. Open squares represent the phosphorylation of pp52 in reactions using ATP. Phosphorylation was negligible for concentrations of ATP and GTP less than 1 μM; these values are not shown.

pseudosubstrate or CAPK catalytic subunit. This suggested that the kinase activity was not dependent on CAPK. Calcium, calmodulin, manganese, and zinc also had no effect on the phosphorylation of pp52. Surprisingly, the addition of cAMP decreased the phosphorylation of pp52 in a concentration-dependent fashion (Figure 15). When cGMP was substituted for cAMP, this effect was not seen.

A 52 kDa protein was also noted to be phosphorylated in the cytosolic S_3 fraction (Figure 11). In contrast to the phosphorylation seen in the apical membrane-rich P_1 fraction, this phosphorylation was observed in both stimulated and unstimulated S_3 fractions, but was more evident in the unstimulated samples. This kinase activity also differed from that seen in the P_1 fraction in its cAMP dependence. Whereas the apical membrane pp52 was phosphorylated in a cAMP-independent fashion and dephosphorylated in a cAMP-dependent manner, the phosphorylation of the cytosolic pp52 required cAMP. The heat-stable CAPK inhibitor and CAPK pseudosubstrate inhibited phosphorylation of the cytosolic pp52, suggesting that this activity was mediated by the catalytic subunit of CAPK. The cytosolic activity also differed from the apical membrane activity in that there was no evidence of endogenous dephosphorylation in the S_3 fraction. These differences suggest either that histamine has different effects on the same protein when it is found in different intracellular compartments or that the 52 kDa proteins found in the membrane and cytosol are two different proteins.

The phosphorylation of the 52 kDa apical membrane protein <u>in vitro</u> suggests that <u>in vivo</u> this protein is actually dephosphorylated by histamine stimulation (Figure 16). If histamine stimulation removes a phosphate from a

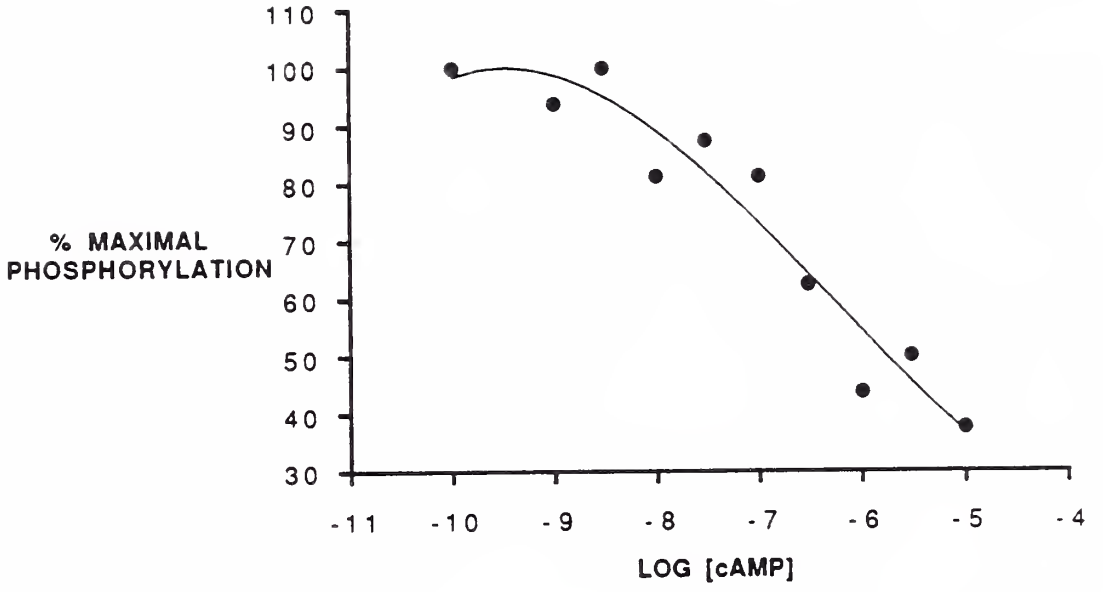

FIGURE 15: The cAMP-dependent dephosphorylation of pp52.
Standard 1 min in vitro phosphorylation reactions were performed in the presence of cAMP (0.1 nM to 10 μM) using protein samples from the maximally stimulated P_1 membrane fraction. Samples were then resolved using SDS-PAGE and autoradiography, and the phosphorylation of pp52 was quantified as described in Chapter 3. Phosphorylation is expressed here as percent maximal phosphorylation.

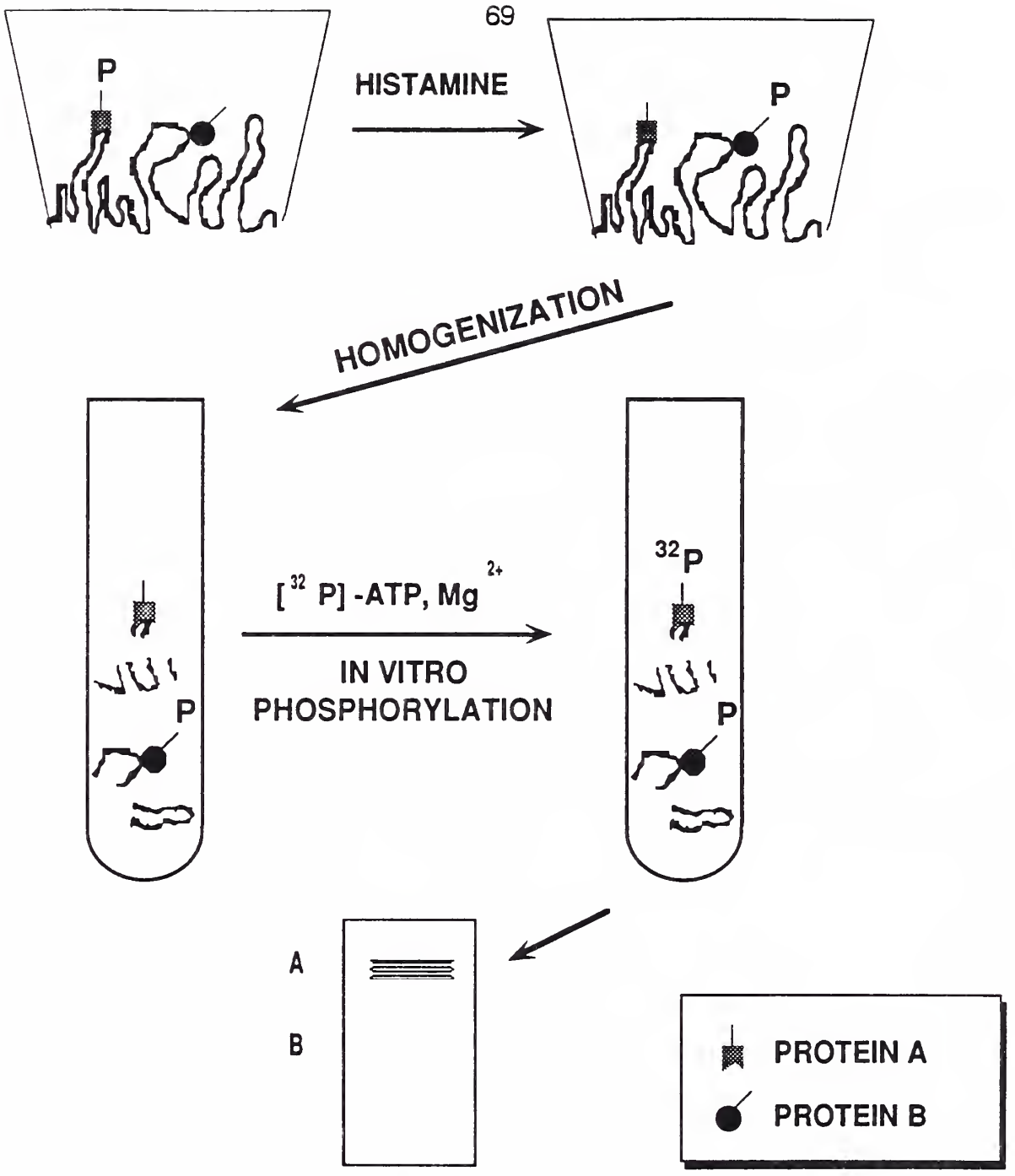

FIGURE 16: The relationship of phosphorylation changes in vivo to phosphorylation changes observed in vitro.
In the resting parietal cell, protein A (gray square) is phosphorylated and protein B (black circle) is not. Histamine stimulation causes the dephosphorylation of protein A and the phosphorylation of protein B with endogenous phosphate (P). The cells are then homogenized. Standard phosphorylation reactions as described in Chapter 3 are performed on the homogenates. Only the dephosphorylated protein A can undergo phosphorylation with the radioactively labelled phosphate (^{32}P). When the sample is resolved using SDS-PAGE and autoradiography, only protein A is detected as a phosphoprotein. Thus the histamine-stimulated dephosphorylation of protein A in vivo is detected as an increase in phosphorylation in vitro. Conversely, an increase in phosphorylation in vivo is detected as a decrease in vitro.

protein, when homogenates are prepared and subjected to in vitro phosphorylation, the empty phosphorylation site will be able to accept a phosphate group. In contrast, a site that is already phosphorylated with native phosphate will not undergo further phosphorylation with $^{32}P_i$. Thus the histamine-stimulated dephosphorylation of a protein in vivo is detected as an increase in phosphorylation in the homogenate. Conversely, a histamine-stimulated increase in phosphorylation in vivo is detected as a decrease in vitro.

The dephosphorylation of apical membrane pp52 observed in the presence of cAMP was somewhat unexpected, since cAMP is known to activate CAPK and thus protein phosphorylation. Further experiments demonstrated that the addition of cAMP activated a phosphatase in the stimulated apical membrane fraction. When cAMP was added to the reaction mixture at the end of the one minute phosphorylation reaction, pp52 was completely dephosphorylated within two minutes (Figure 17). This effect of cAMP was not inhibited by common phosphatase inhibitors such as Zn^{2+}, Mn^{2+}, NaF and pyrophosphate. However it was inhibited by the phosphatase inhibitor okadaic acid[161] in a concentration-dependent fashion (Figure 18). The ID_{50} for okadaic acid was approximately 0.3 μM. This sensitivity to okadaic acid is most consistent with a Type 1 phosphatase activity[162].

IDENTIFICATION OF PP52

Several lines of investigation suggested that both the cytosolic and the apical membrane forms of pp52 could be identified as the regulatory subunit of Type II cAMP-dependent protein kinase (RII). These included isoelectric focusing,

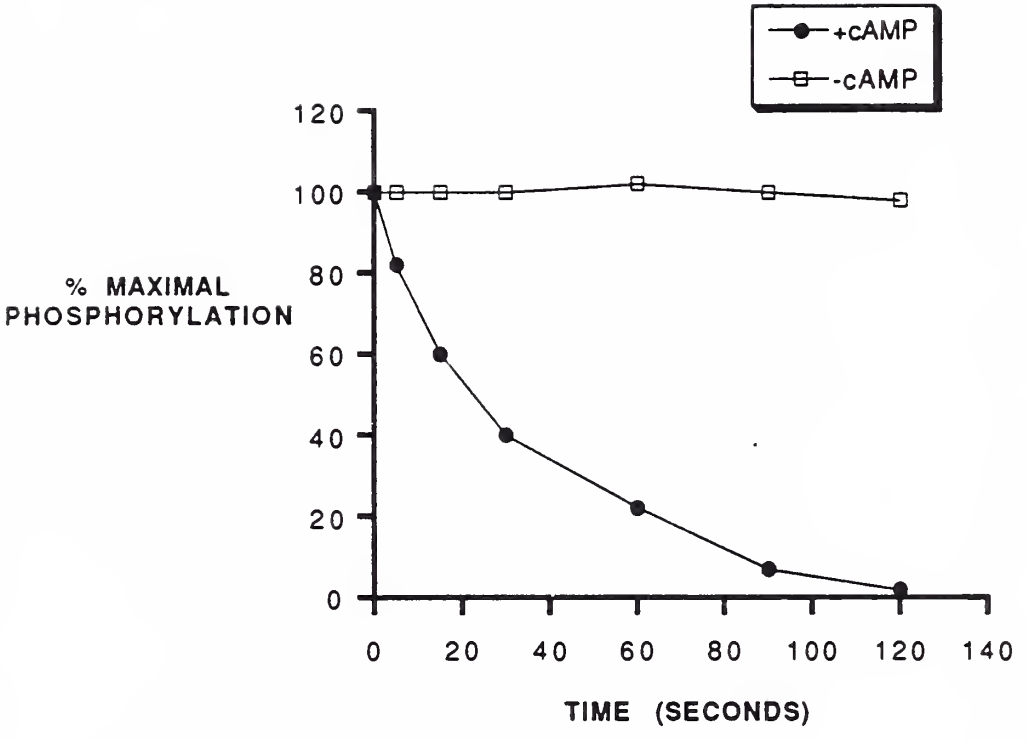

FIGURE 17: Time course of cAMP-dependent dephosphorylation of pp52 in the stimulated apical membrane fraction.
Standard 1 min in vitro protein phosphorylation reactions were performed using protein samples from the maximally stimulated P_1 membrane fraction. At the end of one min 10 mM EDTA was added to stop further phosphorylation. Reactions were then allowed to continue for 0 to 240 sec more in the presence or absence of 10 μM cAMP. Samples were then resolved using SDS-PAGE and autoradiography and the phosphorylation of pp52 was quantified as described in Chapter 3. Phosphorylation is expressed as percent maximal phosphorylation. The time points indicated above were measured from the end of the 1 min reaction. Open squares represent the phosphorylation of pp52 in the absence of cAMP. Closed circles represent the phosphorylation of pp52 in the presence of cAMP. Beyond 120 sec there was no detectable phosphorylation of pp52 in the presence of cAMP; therefore this data is not shown.

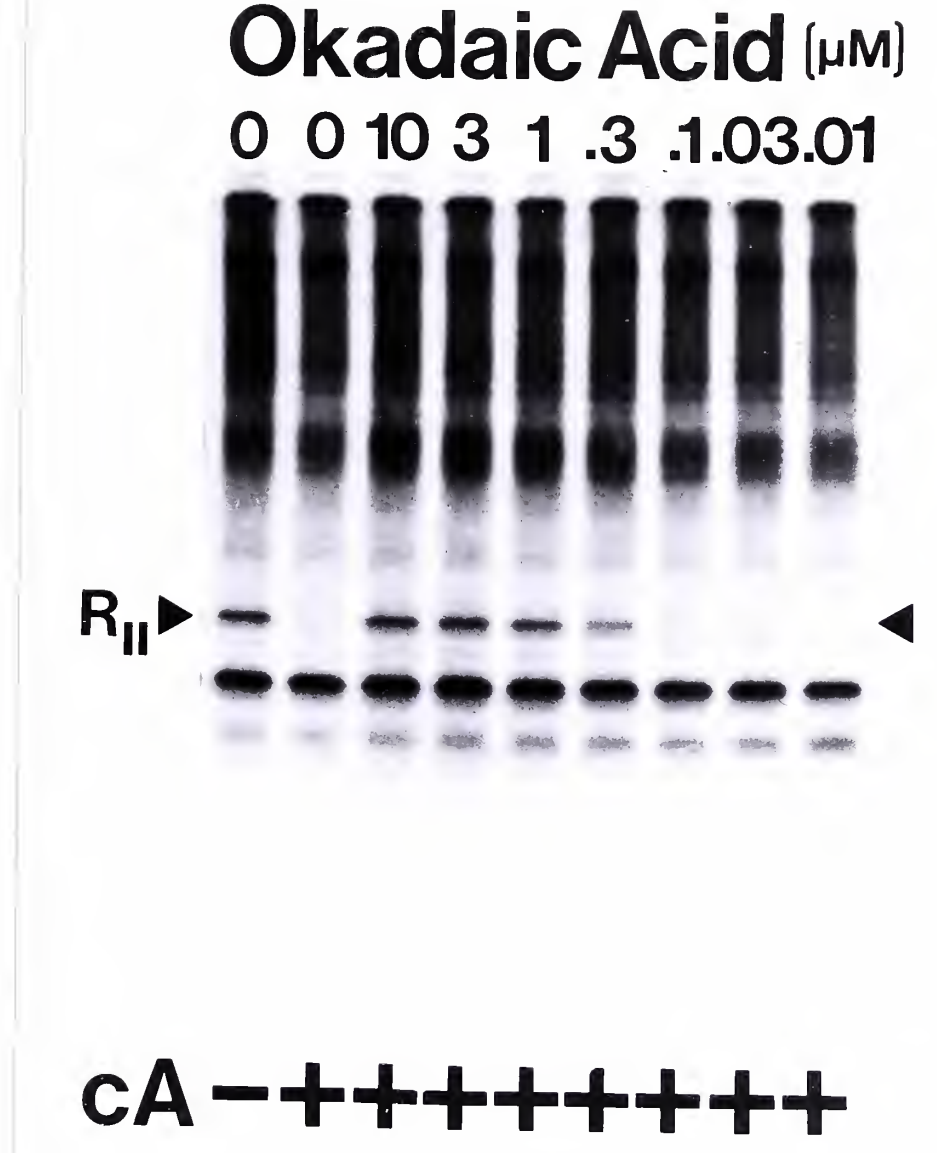

FIGURE 18: Autoradiograph showing the effect of the phosphatase inhibitor okadaic acid on the cAMP-dependent dephosphorylation of pp52.
Standard 1 min in vitro phosphorylation reactions were performed using protein samples from the maximally stimulated P_1 fraction. At one min 10 mM EDTA was added to stop further phosphorylation. At that time cAMP (10 μM) and okadaic acid (1 nM to 10 μM) were added to the reactions, which were allowed to proceed for 3 min more. Samples were then resolved using SDS-PAGE and autoradiography. cA +/- indicates the presence/absence of 10 μM cAMP. The concentration of okadaic acid in each sample is indicated at the top of each lane. The arrow labelled RII marks the location of pp52.

phosphoamino acid analysis, cAMP-binding, and immunoreactivity. Two-dimensional gel electrophoresis showed that both the membrane and cytosolic forms of pp52 had isoelectric points of approximately 5.0 (Figure 19), comparable to published values for RII[163,139]. Phosphoamino acid analysis of the membrane and cytosolic forms of pp52 was also consistent with the conclusion that these proteins are both RII. While RII can be phosphorylated at several sites, the only amino acids that are phosphorylated are serine residues. Similarly, phosphoamino acid analysis showed that pp52 in both membrane and cytosol is phosphorylated on serine residues.

Both forms of pp52 were found to be cAMP-binding proteins. Mammalian cells are believed to contain only two cAMP-binding proteins: the regulatory subunits of Type I and Type II CAPK[164]. 8-azido-[^{32}P]-cAMP binding was used to assay subcellular fractions for cAMP-binding proteins. The P_1 and S_3 fractions each contained two major cAMP-binding proteins (Figure 20). The first, which focused as a doublet of approximately 49 kDa, was identified as RI on the basis of its electrophoretic mobility. Maximal stimulation of parietal cells with histamine and forskolin abolished 8-azido-cAMP binding to this protein. This finding is consistent with results published by Chew[71], who interpreted it as evidence for the stimulation of Type I CAPK. Since the secretagogues induce an increase in intracellular cAMP, this endogenous cAMP is presumably bound to RI, thus preventing 8-azido-cAMP binding and liberating free catalytic subunit. Both P_1 and S_3 also contained cAMP-binding proteins with molecular weights of 52 kDa. Since the molecular weights, as determined by SDS-PAGE, and the isoelectric

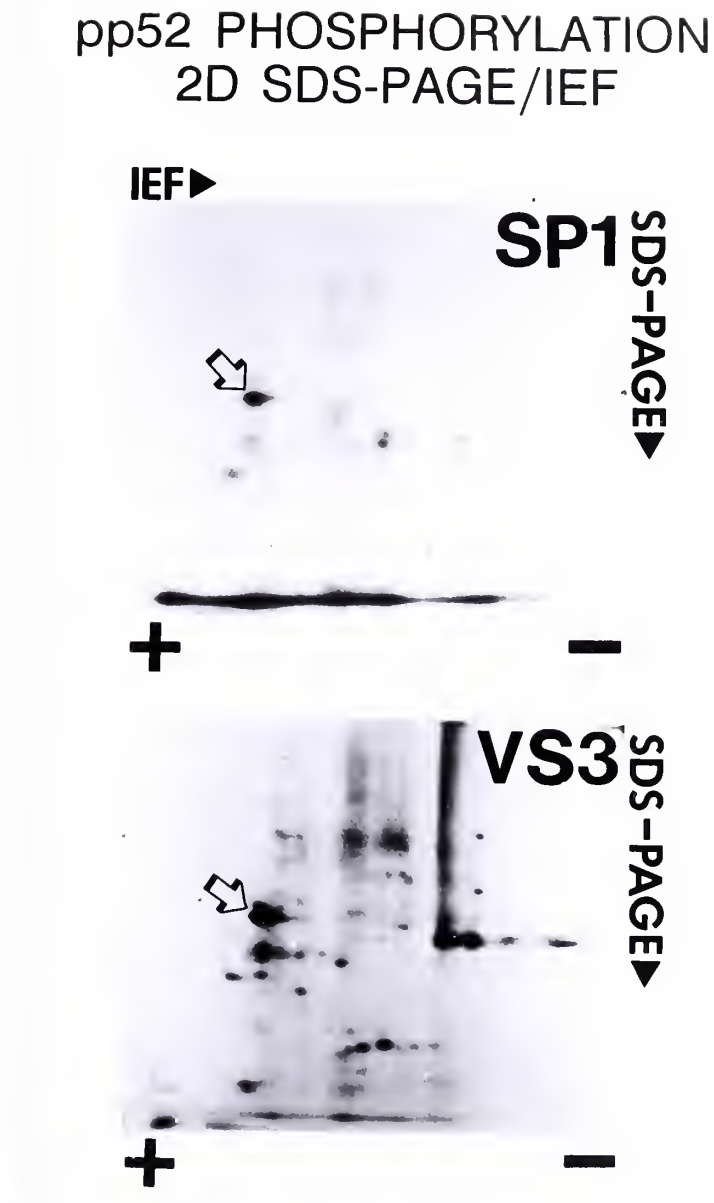

FIGURE 19: Autoradiograph of a two-dimensional gel showing protein phosphorylation in stimulated apical membrane and unstimulated cytosolic fractions. Standard 1 min in vitro phosphorylation reactions were performed using protein samples from maximally stimulated apical membrane fractions (SP_1) and unstimulated cytosolic fractions (VS_3). The phosphorylated samples were then resolved using 2-D gel electrophoresis and autoradiography. Isoelectric focusing was performed in the horizontal dimension; (+) and (-) indicate the acidic and basic ends of the gels. SDS-PAGE was performed in the vertical direction. Arrows mark the positions of the 52 kDa phosphoproteins detected in both fractions. Carbamylated isoelectric focusing standards on the original gel (not shown here) were used to calculate the isoelectric points of these proteins.

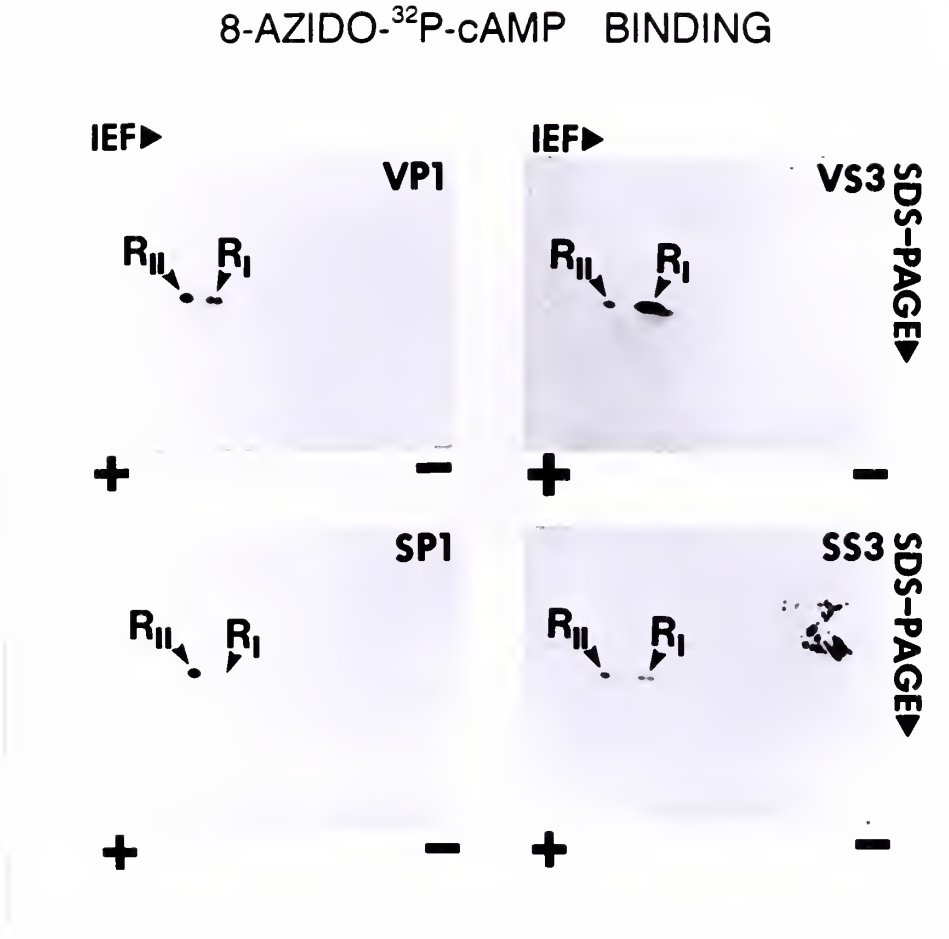

FIGURE 20: Autoradiograph of a two-dimensional gel showing cAMP-binding proteins in apical membrane and cytosolic fractions.
Samples from maximally stimulated (S) and unstimulated (V) P_1 apical membrane and S_3 cytosolic fractions were labeled with 8-azido-[^{32}P]-cAMP as described in Chapter 3. The labeled samples were then resolved using 2-D gel electrophoresis and autoradiography. Isoelectric focusing (IEF) was performed in the horizontal direction; (+) and (-) denote the acidic and basic ends of the gels. SDS-PAGE was performed in the vertical direction. Arrows mark the positions of the 49 kDa cAMP-binding protein thought to be the regulatory subunit of Type I CAPK (RI) and the 52 kDa cAMP-binding protein thought to be the regulatory subunit of Type II CAPK (RII).

points, as determined by two-dimensional gel electrophoresis, were identical with those of the 52 kDa phosphoproteins, it was assumed that the cAMP-binding proteins were in fact the same 52 kDa proteins. The sizes and isoelectric points of these cAMP-binding proteins also agreed with published values for RII. However the distribution of the 49 and 52 kDa cAMP-binding proteins varied between the two subcellular fractions. The smaller cAMP-binding protein predominated in S_3, while the 52 kDa protein was more prominant in the P_1 fraction. Thus it was inferred that RI was the major cAMP-binding protein in cytosol, while RII predominated in the apical membrane fraction.

In stimulated cells, the 52 kDa protein also displayed different patterns of cAMP-binding in the two fractions. In the cytosolic fraction, it behaved much like the 49 kDa protein in that stimulation decreased its 8-azido-cAMP binding. In contrast, in the membrane fraction, stimulation had no effect on the cAMP binding to the 52 kDa protein.

Both forms of pp52 were immunoprecipitated by polyclonal antisera raised against rat heart RII and rat brain RII (Figure 21). However these antisera showed different patterns of immunoreactivity against the membrane and the cytosolic forms of pp52. The antisera against both heart RII and brain RII recognized the cytosolic pp52 equally well. In contrast, the membrane pp52 showed a greater affinity for the antibody against brain RII. From these data, it was concluded that the membrane and the cytosolic forms of pp52 were two different subtypes of RII, the membrane pp52 being more like the neural subtype. This conclusion might explain the fact that phosphorylation of membrane pp52 is independent of cAMP,

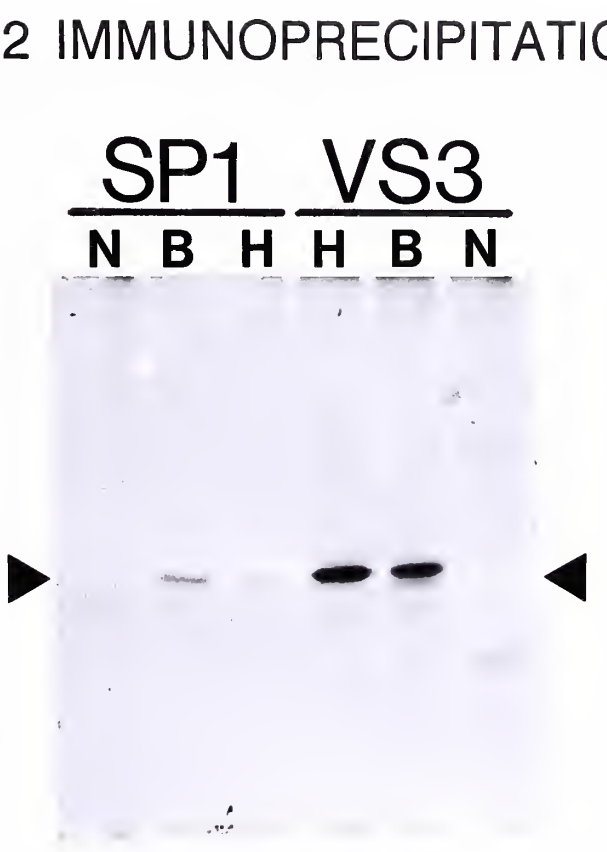

FIGURE 21: Autoradiograph showing immunoprecipitation of pp52 with antibodies against the regulatory subunit of Type II cAMP-dependent protein kinase. Samples from maximally stimulated apical membrane fractions (SP^1) and unstimulated cytosolic fractions (VS^3) were phosphorylated using the standard 1 min in vitro phosphorylation reaction. The phosphorylated samples were then incubated with polyclonal antisera against rat heart RII (H) and rat brain RII (B). Controls were incubated with non-immune rabbit serum (N). After immunoprecipitation as described in Chapter 3, samples were resolved using SDS-PAGE and autoradiography. Arrows indicate pp52.

whereas phosphorylation of cytosolic pp52 is enhanced in the presence of cAMP.

PARIETAL CELL SECRETION STUDIES

Since okadaic acid, a phosphatase inhibitor, prevented the cAMP-dependent dephosphorylation of membrane pp52, the effects of okadaic acid on parietal cell secretion were investigated. Parietal cells were preincubated with okadaic acid for 30 min before the addition of 10 μM histamine. [^{14}C]aminopyrine uptake was used to measure parietal cell acid sequestration, an indirect measure of acid secretion. Okadaic acid alone had no effect on either basal AP uptake or basal levels of intracellular cAMP. However the phosphatase inhibitor caused a dose-dependent decrease in histamine-stimulated AP accumulation (Figure 22). In the presence of 3 μM okadaic acid, histamine-stimulated AP uptake was decreased by 70 %. A similar effect was seen in cells stimulated with 10 μM forskolin and 100 μM histamine, the combination used to maximally stimulate the cells used for subcellular fractionation. To determine whether this effect was mediated by cAMP, cAMP was measured in histamine-stimulated cells. The presence of 3 μM okadaic acid had no effect on the histamine-stimulated increase in cAMP (Figure 23). These data suggested that a protein phosphatase may play a critical role in parietal cell acid secretion.

Additional studies of parietal cell acid secretion were undertaken in the presence of H-8, an inhibitor of cAMP dependent protein kinase with a K_i of 1.2 μM[165]. H-8 did not inhibit histamine-stimulated AP uptake at concentrations ranging from 0.1 μM to 30 μM (Figure 24). This suggested that, contrary to previous

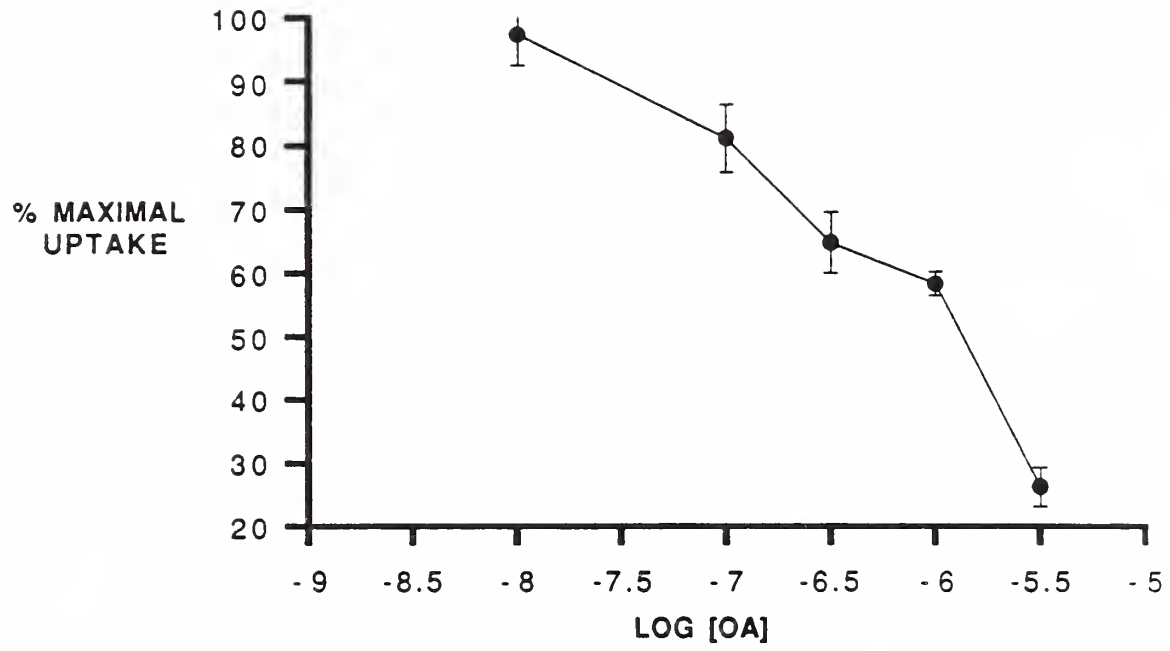

FIGURE 22: The effect of the phosphatase inhibitor okadaic acid on histamine-stimulated [^{14}C]aminopyrine uptake.
Histamine-stimulated [^{14}C]aminopyrine ([^{14}C]AP) uptake was measured in the presence of the phosphatase inhibitor okadaic acid (OA). [^{14}C]AP uptake was measured as described in Chapter 3 and is expressed here as percent maximal uptake ± SEM (n=4). Maximal uptake was determined in the presence of histamine (10 μM) alone. [^{14}C]AP uptake was determined for okadaic acid concentrations from 10 nM to 3 μM. The concentration of histamine was 10 μM for all conditions.

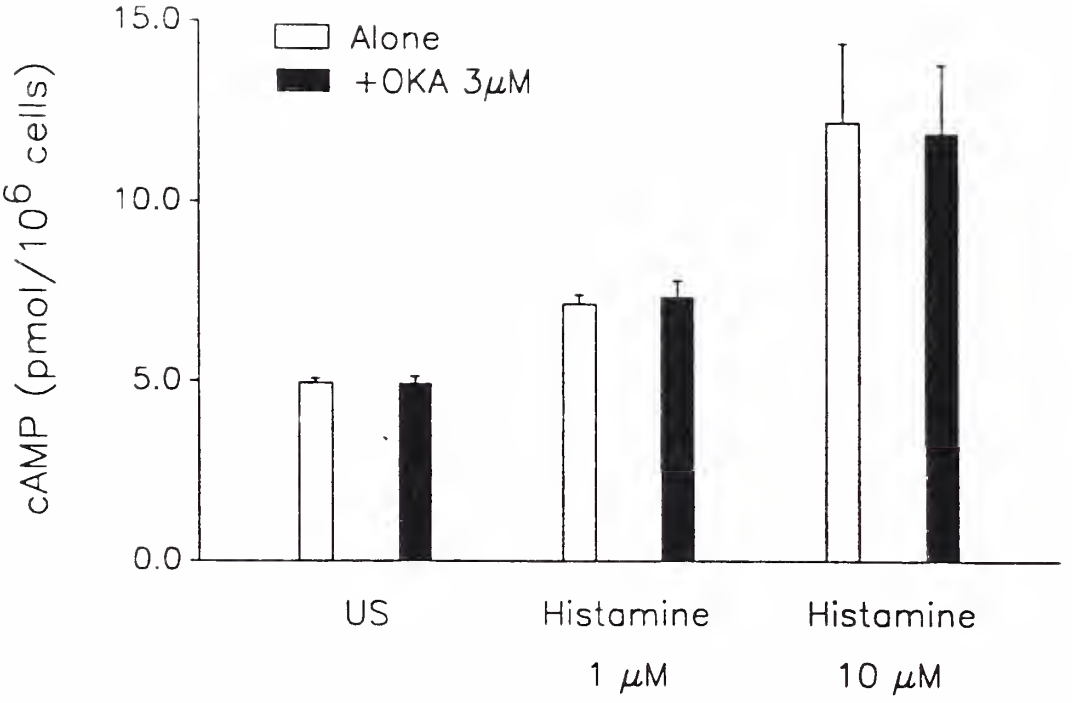

FIGURE 23: The effect of the phosphatase inhibitor okadaic acid on the histamine-stimulated increase in cAMP.
The cellular cAMP content of isolated rabbit parietal cells was measured in the presence and absence of the phosphatase inhibitor okadaic acid (3 μM) as described in Chapter 3. Cellular cAMP content was expressed as pmol/million cells ± SEM (n=4). cAMP levels were measured in unstimulated cells (US), cells stimulated with 1 μM histamine, and cells stimulated with 10 μM histamine. White bars represent the cAMP content of cells in the absence of okadaic acid; dark bars represent the cAMP content of cells in the presence of okadaic acid.

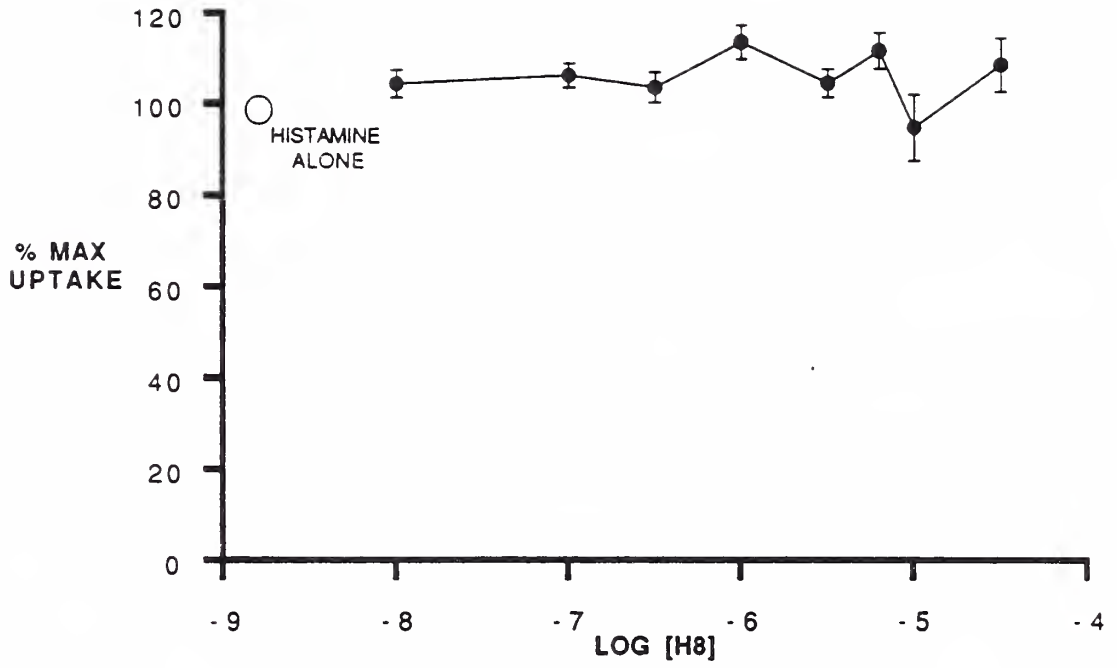

FIGURE 24: The effect of the CAPK inhibitor H-8 on histamine-stimulated [^{14}C]aminopyrine uptake.
Histamine-stimulated [^{14}C]aminopyrine ([^{14}C]AP) uptake in isolated rabbit parietal cells was measured in the presence and absence of the cAMP-dependent kinase inhibitor H-8. [^{14}C]AP uptake was measured as described in Chapter 3 and is expressed here as percent maximal uptake ± SEM (n=4). Maximal uptake was determined in the presence of histamine (10 μM) alone The open circle shows [^{14}C]AP uptake in the presence of histamine (10 μM) alone. The closed circles show the effect of H-8 (10 nM to 30 μM) on histamine (10 μM)-stimulated [^{14}C]AP uptake.

assumptions, activation of CAPK might not be an integral step in histamine-stimulated acid secretion.

CHAPTER 5

DISCUSSION

HISTAMINE-STIMULATED ACID SECRETION: THE CLASSICAL MODEL

Previous work in the parietal cell has assumed that the histamine-stimulated increase in acid secretion was mediated by the cAMP-dependent protein kinase (Figure 25)[69,70,71,72,73,74,75]. Histamine elicited a sustained increase in the intracellular cAMP concentration of the parietal cell[36,37]. Histamine also activated parietal cell CAPK[67,68,69,70,71]. These data led to the assumption that CAPK mediated histamine-stimulated acid secretion, presumably by phosphorylating key protein intermediates.

HISTAMINE-STIMULATED ACID SECRETION: A NEW MODEL

The data presented here suggest that while secretion still appears to be mediated by cAMP, the cyclic nucleotide does not act solely via CAPK, since the addition of the CAPK inhibitor H-8 to whole parietal cells did not affect acid secretion. At least one other investigator has reported a similar finding, in which cAMP-dependent exocytosis did not appear to be mediated by CAPK[99].

Our data demonstrate that histamine stimulation of rabbit parietal cells affects the phosphorylation state of a 52 kDa protein that is found in the apical membrane-rich fraction. Both kinase and phosphatase activities in this fraction have been characterized. Furthermore, the 52 kDa substrate has been identified as the neural subtype of the regulatory subunit of Type II CAPK. Okadaic acid, a phosphatase inhibitor, inhibits both the dephosphorylation of this protein and the

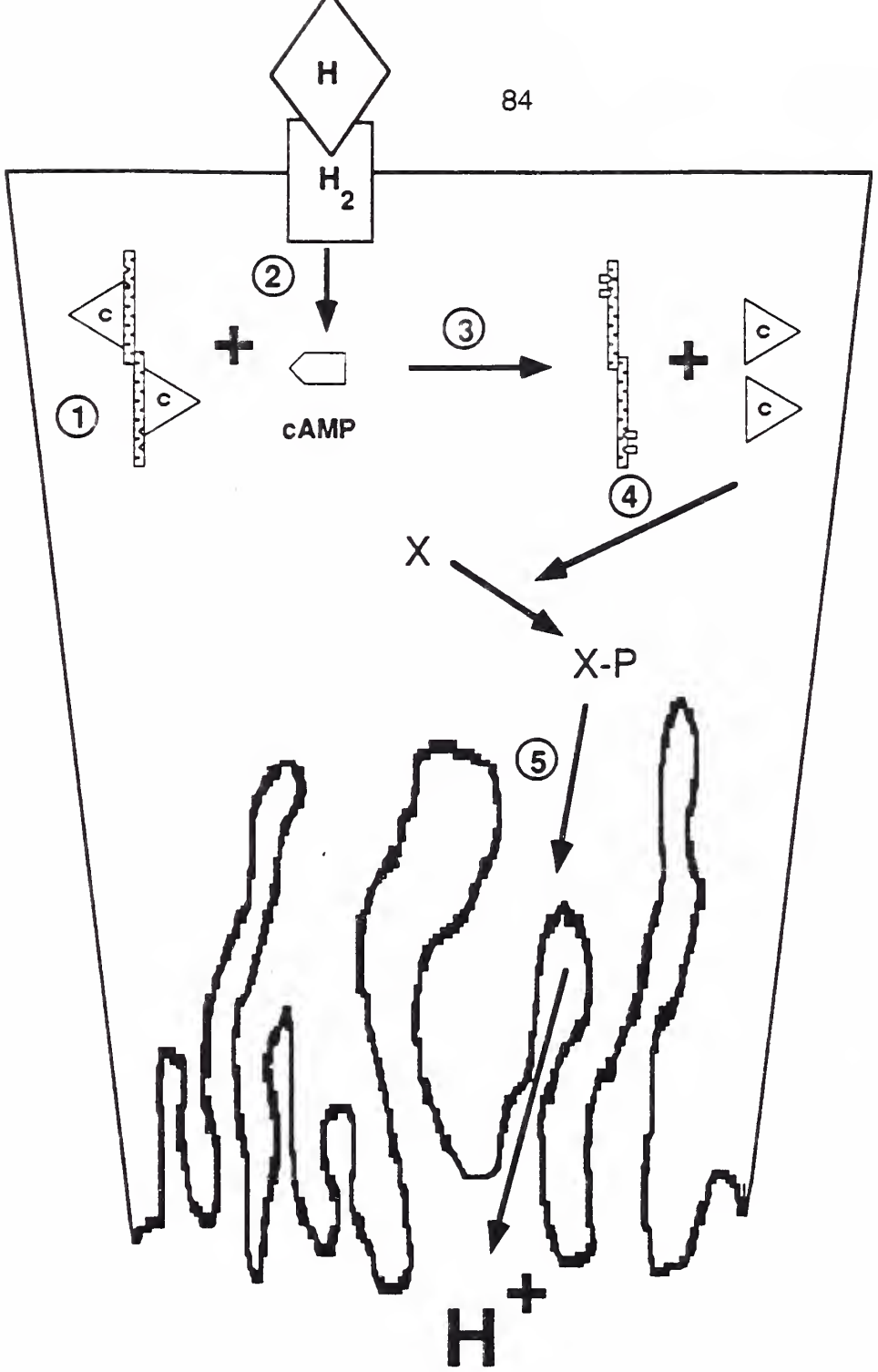

FIGURE 25: Histamine-stimulated acid secretion: the classical model.
1) In the resting cell, cAMP-dependent kinase (CAPK) is found in the inactive form. 2) Histamine stimulation increases the intracellular concentration of cAMP. 3) cAMP binds to the regulatory subunit of CAPK, releasing active catalytic subunit. 4) The free catalytic subunit phosphorylates protein X. 5) The phosphorylated protein acts as an intermediary in histamine-stimulated acid secretion. H - histamine; H_2 - type 2 histamine receptor; C - catalytic subunit of CAPK; X - a protein; X-P - the phosphorylated form of the protein.

secretion of acid by the parietal cell. This suggests that a phosphatase plays a key role in histamine-stimulated acid secretion.

These data have led us to propose a new model for histamine-stimulated parietal cell acid secretion (Figure 26). 1) In the resting state, RII is phosphorylated by a kinase that has not yet been identified. 2) Histamine stimulation increases the concentration of intracellular cAMP. 3) Cylic AMP binds to apical membrane RII, changing its conformation. 4) This change in conformation makes a phosphate group accessible to an apical membrane phosphatase that dephosphorylates RII. 5) The dephosphorylated RII promotes acid secretion by an unknown mechanism.

It is possible that cAMP directly or indirectly acts on the phosphatase rather than the substrate. However since RII is known to be a cAMP-binding protein it is more likely that the effect of cAMP is substrate-mediated. The physiological role of this phosphorylation change is not yet known. Since previous data have shown RII to be associated with cytoskeletal elements[143,146], RII may influence the cytoskeletal changes associated with parietal cell acid secretion[21,22,23]. The dephosphorylation of apical membrane RII may activate CAPK and directly or indirectly cause the phosphorylation of cytoskeletal elements. However other investigators have found that Type II CAPK is not activated in histamine-stimulated parietal cells[71]. It is therefore possible that the apical membrane RII interacts with the cytoskeleton through a function other than its regulation of CAPK.

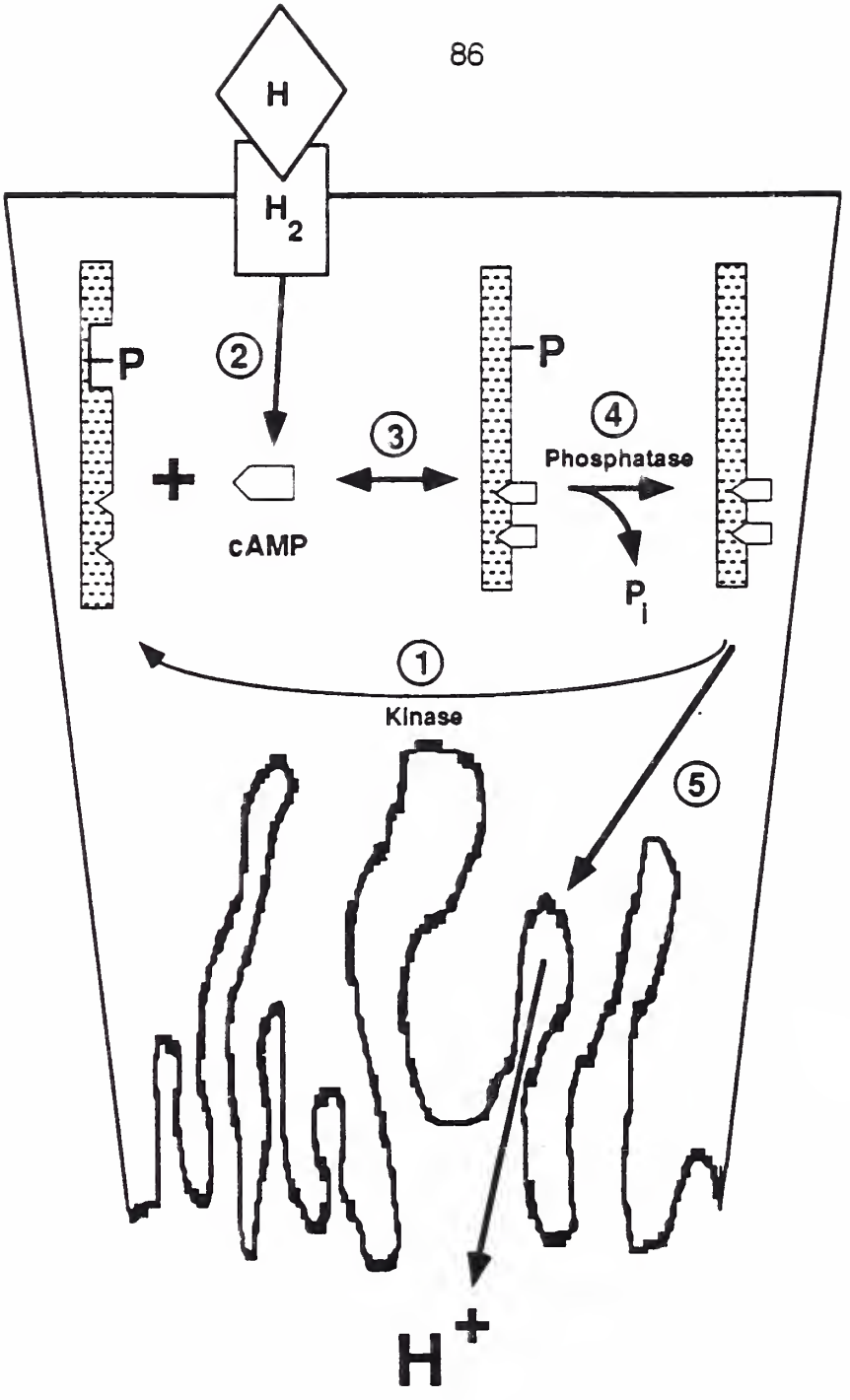

FIGURE 26: Histamine-stimulated acid secretion: a new model.
1) In the resting state, a membrane-bound kinase phosphorylates the apical membrane RII. 2) Histamine stimulation increases the intracellular concentration of cAMP. 3) cAMP binds to RII, changing its conformation. 4) This change in conformation makes the phosphate group accessible to an apical membrane phosphatase. 5) The dephosphorylated RII acts as an intermediary in histamine-stimulated acid secretion. H - histamine; H_2 - type 2 histamine receptor; P - phosphate.

Identification of the phosphatase

Several phosphatases have been reported to cause the cAMP-dependent dephosphorylation of RII[166,127,124,133,167]. These generally require cAMP concentrations between 4 and 25 μM, close to the concentration used in our experiments. However our data characterizing the apical membrane phosphatase activity differed from previously published reports, suggesting that there may be more than one cAMP-dependent phosphatase that acts on RII. Most of the cAMP-dependent phosphatases described in the literature are inhibited by phosphatase inhibitors such as NaF and $ZnCl_2$. These agents did not affect the activity of the apical membrane phosphatase in our preparation. This difference may also reflect differences in the substrate. In at least one instance, the phosphatase was probably acting on the non-neural form of RII, since dephosphorylation altered its electrophoretic mobility[133]. In contrast, our data suggest that the phosphatase substrate in the apical membrane fraction is probably the neural form of RII.

While most of the phosphatases that have been reported to act on RII show cAMP dependence, Blumenthal et al. have described an interesting calmodulin-dependent phosphatase that catalyzes the removal of a phosphate group from the autophosphorylated serine-95 of RII[168]. This phosphatase, also known as calcineurin, has been reported to bind tightly to Type II CAPK from brain via the regulatory subunit[169]. As mentioned earlier, RII is known to interact with proteins other than the regulatory subunit of CAPK[143,144,145]. It is unlikely that this phosphatase is the same as that identified in the apical membrane fraction of stimulated parietal cells. The calmodulin-dependent phosphatase activity was completely

inhibited by 2 mM EDTA, whereas the dephosphorylation of RII in the apical membrane was completely unaffected by the addition of 10 mM EDTA.

Although there are no other reports of histamine-stimulated phosphatases in the parietal cell, Im et al. have identified a phosphatase in membranes prepared from the stomachs of carbachol-injected rats[170]. Like the P_1 fraction used in our experiments this membrane preparation was enriched in the H^+/K^+-ATPase. However unlike the histamine-stimulated phosphatase described here, the carbachol-stimulated phosphatase was inhibited by NaF, $ZnCl_2$, and pyrophosphate. The carbachol- and histamine-stimulated phosphatase activities are therefore unlikely to be the result of the same enzyme.

Although a number of phosphatases that act on RII have been described, most of these descriptions are not compatible with the data from the rabbit parietal cell. This may reflect the fact that these phosphatases act on a different phosphorylation site and/or subtype of RII. However Kiss et al. have identified a CAPK catalytic subunit-independent kinase and a cAMP-dependent phosphatase that act on RII in rat liver membranes. These appear to be similar to the activities seen in parietal cell membranes134. The addition of NaF did not inhibit phosphatase activity in either preparation. Furthermore, in both cases the site of phosphorylation and dephosphorylation appeared to be a site other than the autophosphorylation site of RII.

Several pieces of evidence support the conclusion that the phosphorylation site of interest on apical membrane RII is not the autophosphorylation site. First, the autophosphorylation of serine-95 has a $K_{½}$ for ATP of approximately 0.4 μM[123].

The phosphorylation of the apical membrane RII has a $K_{½}$ that is approximately tenfold higher. Second, the addition of CAPK catalytic subunit does not increase the phosphorylation of the site on the apical membrane RII, as would be expected if it were the autophosphorylation site. Other sites on RII that are known to be phosphorylated are the serines-44 and-47 phosphorylated by glycogen synthase kinase 3 (GSK 3)[128], and the serines-74 and -76 phosphorylated by casein kinase II (CK II)[129]. However immunoprecipitation with antibodies against heart and brain RII has shown that the apical membrane RII more closely resembles the neural form of RII, which does not possess the consensus sequence for phosphorylation by GSK 3[142]. While it is possible that the apical membrane RII is phosphorylated at a site that has not been previously described, the data also support the possibility that the apical membrane RII is phosphorylated and dephosphorylated at the CK II site.

The phosphatase activity seen in apical membranes from stimulated rabbit parietal cells therefore seems to be a type I phosphatase that removes a phosphate group from the CK II site of RII. Like the activity described by Kiss et al.[170], this phosphatase is dependent on cAMP but not inhibited by NaF.

Identification of the kinase

In the resting state, the apical membrane RII presumably returns to the phosphorylated state. It is not known which kinase mediates this reaction. The catalytic subunit of CAPK is unlikely to be responsible, since it is thought to phosphorylate only serine-95. It is possible that CK II phosphorylates the apical

membrane RII. CK II is a ubiquitous protein kinase found in many different organisms and species[171]. Despite its widespread occurrence and ability to phosphorylate a number of different substrates in vitro, it has as yet no known physiological substrates. The kinase that phosphorylates the apical membrane RII exhibits several features that make CK II a likely candidate. Like CK II, the apical membrane RII kinase is able to use both ATP and GTP as phosphate donors but has a greater affinity for ATP[172]. Furthermore, both kinases are also active over a wide pH range. While these data do not conclusively identify the apical membrane RII kinase, it is possible that RII will be the first substrate reported to be phosphorylated by CK II under physiological conditions.

Other forms of RII in the parietal cell

The form of RII identified in the cytoplasm of rabbit parietal cells does not appear to be a substrate for the kinase and phosphatase that act on the apical membrane RII. Based on their immunoreactivity, we have hypothesized that the membrane RII and the cytosolic RII are two different subtypes of RII. The former appears to resemble the neural form, while the latter is probably closer to the non-neural variety. This hypothesis might explain why histamine stimulation of rabbit parietal cells affects the membrane and cytosolic forms of RII differently. An analogous situation has been observed in Friend erythroleukemic cells[137]. When these cells were stimulated to differentiate, one form of RII increased while the other was unaffected. This difference in response may reflect modifications to the two forms of RII that make them react differently to stimuli. However it may also

reflect the subcellular distribution of RII subtypes. The different forms of RII may be found in separate intracellular compartments, and individual stimuli may exert their effects only at selected locations within the cell.

CONCLUSION

The data presented here support a new model for histamine-stimulated parietal cell acid secretion in which the phosphorylation state of a neural-type RII in the apical membrane plays a critical role. Whole cell secretion data indicate that a phosphatase activity is essential to histamine-stimulated acid secretion. Histamine stimulation of isolated rabbit parietal cells appears to activate a membrane-associated type 1 phosphatase that dephosphorylates the apical membrane RII. The apical membrane also contains a kinase that phosphorylates the membrane-associated RII. The data indicate that this kinase may be casein kinase II. While the phosphorylation site of the apical membrane RII has not been conclusively identified, the data suggest that this may be the site phosphorylated by casein kinase II *in vitro*. The parietal cell also contains a cytosolic form of RII that is not a substrate for the histamine-stimulated apical membrane phosphatase. The cytosolic RII appears to resemble the non-neural subtype of RII.

FUTURE DIRECTIONS

While this work provides some insight into the molecular mechanism of acid secretion, a number of key questions remain to be answered. First, it must be conclusively demonstrated in whole cell experiments that histamine stimulation

leads to the dephosphorylation of RII. Second, the RII subtypes in the parietal cell must be purified and characterized. Third, the phosphorylation site on apical membrane RII needs to be specifically identified and its biochemical function understood. Fourth, the apical membrane kinase and phosphatase must be purified and identified. Fifth, it must be determined whether apical membrane RII exerts its effects through CAPK or whether it is associated with other effector proteins. Lastly, it must be seen whether other secretagogues that promote acid secretion, such as carbachol and gastrin, are also able to affect the phosphorylation state of RII.

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